REPORT DOCUMENTATION PAGE

Form Approved OMB NO. 0704-0188

1. REPORT DATE (DD-MM-YYYY)	2. REPORT TYPE	3. DATES COVERED (From - To)
08-12-2010	Final Report	17-Sep-2007 - 16-Sep-2010

4. TITLE AND SUBTITLE
Regulation of initial attachment of P. aeruginosa

5a. CONTRACT NUMBER
W911NF-07-1-0604

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER
611102

6. AUTHORS
Dr. Karin Sauer

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAMES AND ADDRESSES
Research Foundation of SUNY at Binghamton
Research Development Services
The Research Foundation of SUNY, Binghamton University
Binghamton, NY 13902 -

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Research Office
P.O. Box 12211
Research Triangle Park, NC 27709-2211

10. SPONSOR/MONITOR'S ACRONYM(S)
ARO

11. SPONSOR/MONITOR'S REPORT NUMBER(S)
53295-LS.1

12. DISTRIBUTION AVAILIBILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES
The views, opinions and/or findings contained in this report are those of the author(s) and should not contrued as an official Department of the Army position, policy or decision, unless so designated by other documentation.

14. ABSTRACT
The goal of this proposal is to identify biofilm-specific regulatory proteins involved in the regulation of initial biofilm formation that once inactivated, impair or arrest the biofilm formation process. We have identified 8 regulatory proteins which become sequentially phosphorylated/dephopshorylated during biofilm formation. Inactivation of sagS and bfiS arrested biofilm formation prior to the maturation-1 stage of biofilm development, as indicated by analyses of biofilm architecture, and protein and phosphoprotein patterns. Discontinuation of sagS and

15. SUBJECT TERMS
biofilm development, phosphorylation, phosphorelay, two-component regulatory systems, small RNAs, ribonuclease G, virulence, small regulatory RNAs rsmYZ, intracellular signaling molecule cyclic di-GMP, antibiotic resistance, differential expression of genes

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	15. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			Karin Sauer
UU	UU	UU	UU		19b. TELEPHONE NUMBER: 607-777-3157

Report Title

Regulation of initial attachment of P. aeruginosa

ABSTRACT

The goal of this proposal is to identify biofilm-specific regulatory proteins involved in the regulation of initial biofilm formation that once inactivated, impair or arrest the biofilm formation process. We have identified 8 regulatory proteins which become sequentially phosphorylated/dephopshorylated during biofilm formation. Inactivation of sagS and bfiS arrested biofilm formation prior to the maturation-1 stage of biofilm development, as indicated by analyses of biofilm architecture, and protein and phosphoprotein patterns. Discontinuation of sagS and bfiS expression in established biofilms resulted in the collapse of biofilms to an earlier developmental stage indicating a requirement for these regulatory systems for the development and maintenance of normal biofilm architecture. plkR inactivation did not affect biofilm formation while plkR overexpression coincided with dispersion. Downstream gene targets of selected regulators were identified. Inactivation of bfiS coincided with decreased virulence. Inactivation of sagS rendered biofilms as susceptible to antimicrobials as planktonic cells indicating that biofilm resistance is regulated. Findings from this research indicated biofilm formation to be governed by a previously unidentified regulatory program, and provided detailed information how to control or prevent biofilm growth by interrupting the sequence of gene activation and affecting a cell's ability to attach to a surface and form deleterious biofilms.

List of papers submitted or published that acknowledge ARO support during this reporting period. List the papers, including journal references, in the following categories:

(a) Papers published in peer-reviewed journals (N/A for none)

*Petrova, O.E., and K. Sauer. 2009. A Novel Signaling Network Essential for Regulating Pseudomonas aeruginosa Biofilm Development. PLoS Pathogens 5: e1000668.

** Petrova, O.E., and K. Sauer. 2010. The novel two-component regulatory system BfiSR regulates biofilm development directly by controlling the small RNA rsmZ through CafA. J. Bacteriol. 192:5275-5288.

* This publication received some media attention and excerpts were featured on the following websites:

- http://www.sciencecodex.com/researchers_discover_new_ways_to_treat_chronic_infections
- http://www.sciencedaily.com/releases/2009/12/091218151327.htm
- http://www.healthjockey.com/2010/01/02/development-of-biofilms-may-lead-to-new-ways-for-treating-chronic-infections/
- http://www.britannica.com/bps/additionalcontent/18/47256242/A-Novel-Signaling-Network-Essential-for-Regulating-Pseudomonas-aeruginosa-Biofilm-Development

** This publication was highlighted by a commentary in the Journal of Bacteriology (Goodman, 2010) as well by a journal highlight in the ASM journal Microbe (http://www.microbemagazine.org/index.php/10-2010-journal-highlights/2913-improved-understanding-of-regulation-of-biofilm-formation)

Number of Papers published in peer-reviewed journals: 2.00

(b) Papers published in non-peer-reviewed journals or in conference proceedings (N/A for none)

Number of Papers published in non peer-reviewed journals: 0.00

(c) Presentations

Number of Presentations: 0.00

Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Oral presentations, invited
09/2010 Regulatory systems required for biofilm development and the maintenance of the planktonic mode of growth. Biofilms IV, 4th International SFAM meeting on Biofilms, Winchester, UK.

07/2010 Biofilm Development and Dispersion. SFAM Summer conference, Brighton, UK.

04/2010 Signaling networks essential for regulating Pseudomonas aeruginosa biofilm development, University of Oklahoma Health Sciences Center, Oklahoma City, OK.

04/2010 Signaling networks essential for regulating Pseudomonas aeruginosa biofilm development, Department of Microbiology, Montana State University, Bozeman, MT.

03/2010 A novel signaling network essential for regulating Pseudomonas aeruginosa biofilm development. Mathematical Biosciences Institute (MBI) workshop, Columbus, OH.

11/2009 A novel signaling network essential for regulating Pseudomonas aeruginosa biofilm development. 5th ASM (American Society for Microbiology) Biofilms conference, Cancun, Mexico

10/2009 A novel signaling network essential for regulating Pseudomonas aeruginosa biofilm development. Syracuse University, Syracuse, NY.

04/2009 Regulation of biofilm formation by Pseudomonas aeruginosa. Stevens Institute of Technology, Department of Engineering, Hoboken, NJ.

02/2009 Regulation of biofilm formation by Pseudomonas aeruginosa. University of Rochester, Department of Microbbiology and Immunology, Rochester, NY.

10/2008 Using tags and isotopes to track post-translational modifications during biofilm formation by Pseudomonas aeruginosa. Binghamton University, Department of Chemistry, Binghamton, NY.

Poster presentations at national and international conferences
11/2009 * Petrova, O., K. Sauer. Novel regulatory systems responsible for maintaining the planktonic lifestyle of the opportunistic human pathogen Pseudomonas aeruginosa. 5th ASM (American Society for Microbiology) Biofilms conference, Cancun, Mexico

09/2009 ** Petrova, O., K. Sauer. Coordinated signaling events regulate transition from initial attachment to maturation during Pseudomonas aeruginosa biofilm formation. Eurobiofilms 2009, Rome, Italy.

05/2009 Petrova, O., K. Sauer. Pseudomonas aeruginosa biofilm development is coordinated by a sequential series of signal transduction events. ASM conference, 109th General meeting, Philadelphia, PA.

07/2008 Petrova, O., G. Vazquez, and K. Sauer.Pseudomonas aeruginosa biofilm development is coordinated by multiple stage-specific transcriptional regulators. Corning Incorporated Science & Technology, Elmira, NY.

05/2008 Petrova, O., G. Vazquez, and K. Sauer. Pseudomonas aeruginosa biofilm development is coordinated by multiple stage-specific transcriptional regulators. ASM conference, 108th General meeting, Boston, MA.

Number of Non Peer-Reviewed Conference Proceeding publications (other than abstracts): 0

Peer-Reviewed Conference Proceeding publications (other than abstracts):

Number of Peer-Reviewed Conference Proceeding publications (other than abstracts): 0

(d) Manuscripts

Manuscripts in preparation

Marques, C, O.E. Petrova, Y. Li, and K. Sauer. Antimicrobial resistance and virulence are developmental aspects of biofilm formation by the human pathogen Pseudomonas aeruginosa.

Petrova, O.E., and K. Sauer. SagS contributes to the sessile-motile switch by the human pathogen Pseudomonas aeruginosa

Petrova, O.E., and K. Sauer. Role of the transcriptional regulator PlkR in attachment and biofilm dispersion.

Number of Manuscripts: 3.00

Patents Submitted

Patents Awarded

Awards

Graduate Students

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Names of Post Doctorates

NAME	PERCENT_SUPPORTED
Olga E. Petrova	1.00
Gustavo Vazquez	1.00
Dr. Claudia Marques	0.50
Dr. Joanne Coulburn	0.50
FTE Equivalent:	**3.00**
Total Number:	**4**

Names of Faculty Supported

NAME	PERCENT_SUPPORTED	National Academy Member
Dr. Karin Sauer	0.25	No
FTE Equivalent:	**0.25**	
Total Number:	**1**	

Names of Under Graduate students supported

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Student Metrics

This section only applies to graduating undergraduates supported by this agreement in this reporting period

The number of undergraduates funded by this agreement who graduated during this period:	0.00
The number of undergraduates funded by this agreement who graduated during this period with a degree in science, mathematics, engineering, or technology fields:.....	0.00
The number of undergraduates funded by your agreement who graduated during this period and will continue to pursue a graduate or Ph.D. degree in science, mathematics, engineering, or technology fields:.....	0.00
Number of graduating undergraduates who achieved a 3.5 GPA to 4.0 (4.0 max scale):	0.00
Number of graduating undergraduates funded by a DoD funded Center of Excellence grant for Education, Research and Engineering:	0.00
The number of undergraduates funded by your agreement who graduated during this period and intend to work for the Department of Defense	0.00
The number of undergraduates funded by your agreement who graduated during this period and will receive scholarships or fellowships for further studies in science, mathematics, engineering or technology fields:	0.00

Names of Personnel receiving masters degrees

NAME
Total Number:

Names of personnel receiving PHDs

NAME
Olga E. Petrova
Total Number: 1

Names of other research staff

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Sub Contractors (DD882)

Inventions (DD882)

Title:	Regulation of initial attachment of *P. aeruginosa*
Grant number:	W911NF0710604
P.I.:	Karin Sauer
Grantee Institution:	The Research Foundation of SUNY, Binghamton University Vestal Parkway East – PO Box 6000 Binghamton, NY 13902-6000
Funding period:	08/2007-09/2010

FINAL REPORT

The goal of this proposal is to identify biofilm-specific regulatory proteins involved in the regulation of initial biofilm formation that once inactivated, impair or arrest the biofilm formation process. We therefore proposed three specific aims:

Aim 1. Identify (regulatory) proteins that are unique to the transition from planktonic growth stage to initial attachment and maturation-1 biofilm developmental stages.Aim 2. Determine the role of regulatory proteins in biofilm development. Aim 3. Determine susceptibility of mutants impaired in the progression of biofilm developmental to antimicrobial treatment.

Specific Aim 1: Identify (regulatory) proteins that are unique to the transition from planktonic growth stage to initial attachment and maturation-1 biofilm developmental stages.

The goal of Specific Aim 1 was to identify (regulatory) proteins that are unique to the transition from planktonic growth stage to initial attachment and maturation-1 biofilm developmental stages. This aim has now been completed. A summary of our findings is listed below.

The phosphoprotome of three developmental stages (reversibly and irreversibly attached cells, maturation-1) was analyzed for the presence of planktonic- and attachment-specific phosphorylation events. Two procedures were used to do so, a combination of 2D/PAGE and immunoblot analysis and purification of phosphorylated proteins by MOAC and subsequence detection using cICAT labeling.

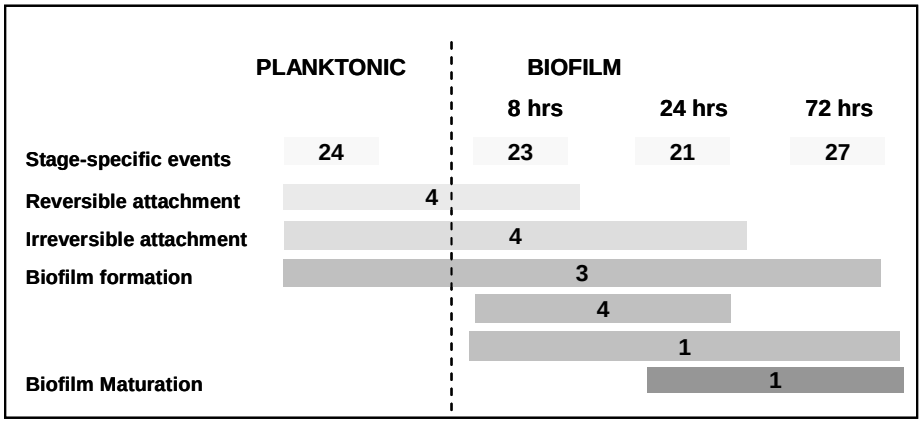

Fig. 1. Detection of proteins phosphorylation events. Protein extracts were obtained from planktonic and biofilm cells following 8, 24, and 72 hr of growth under flowing conditions. Proteins were first separated by 2D/PAGE and subsequently subjected to immunoblot analysis. Phosphorylation events are subcategorized according to their appearance over the course of biofilm development as being stage-specific (phosphorylation events that were only detected at one growth stage, e.g. planktonic cells or 8h old biofilms, biofilm-specific (protein phosphorylation only detected in biofilm cells regardless of their age). Furthermore, protein phosphorylation events that occurred at different stages over the course of biofilm formation are subcategorized as reversible and irreversible attachment, and biofilm formation. The number per bar indicate the number of protein phosphorylation events detected per (sub)category.

We detected protein phosphorylation events specific to the planktonic growth stage, the biofilm mode of growth (constitutive phosphorylation regardless of age of the biofilm) and protein phosphorylation events specific to the developmental stage of the biofilm (Fig. 1).

Phosphorylated proteins were identified by LC-MS-MS analysis. Regulatory proteins that were found to be phosphorylated are listed in Table 1.

Table 1. Identification of regulatory proteins that are differentially phosphorylated over the course of *P. aeruginosa* biofilm formation.

Protein ID				Protein Description			ID Method
Plk	Biofilm (hr)						
	8	24	72				
√	-	-	-	PA0701		probable transcriptional regulator	LC-MS/MS
√	-	-	-	PA1128		probable transcriptional regulator	LC-MS/MS
√	-	-	-	PA1504		probable transcriptional regulator	LC-MS/MS
√	-	-	-	PA2824		probable sensor/response regulator hybrid	LC-MS/MS
√	√	√	√	PA2047		probable transcriptional regulator	IP/IB
-	√	√	√	PA4197	*bfiS*	probable two-component sensor	IP/IB
-	√	√	√	PA0928	*gacS*	sensor/response regulator hybrid	IP/IB; LC-MS/MS
-	-	√	√	PA2096		probable transcriptional regulator	LC-MS/MS

[a] √, protein phosphorylation detected by immunoblot analysis and/or LC-MS-MS analysis in conjunction with cICAT labeling.

Plk, phosphorylation events detected in cell extracts obtained from cells grown planktonically.

IP/IB, Proteins were obtained following immunoprecipitation and 2D/PAGE (IP) while protein phosphorylation was confirmed by immunoblot analysis using anti-Phospho-(Ser/Thr)Phe antibodies (IB).

To determine whether phosphorylation coincided with *de novo* gene expression or reflected biofilm-specific patterns of posttranslational modification, RT-PCR was used. *PA4197* and *PA2824* were constitutively expressed regardless of the mode of growth (not shown). PA0701 expression was detected to be planktonic-specific (not shown).

Specific Aim 2: Determine the role of regulatory proteins in biofilm development.

The goal of Specific aim 2 (proposed to be initiated in year 2) is to determine the role of regulatory proteins in biofilm development.

To this date, we have generated deletion mutants in the following regulatory proteins: PA4197 (bfiS), PA0928 (gacS), PA0701 (plkR), and PA2824 (sagS). The mutants were selected based on their timing of phopshorylation over the course of biofilm formation. PA0701 and PA2824 are uniquely phosphorylated in planktonic cells (see Table 1), while PA0928 and PA4197 are phosphorylated in early biofilms. In addition, we have complemented each mutant by cloning the wild type gene(s) into the plamid pJN105 under the control of an arabinose inducible promoter. Each construct was then transferred by mating into the respective mutant (complementation) and PAO1 wild type strain (overexpresser strain).

Role of regulatory proteins in fitness, initial attachment, motility, and polysaccharide production.

To determine the role of phosphorylated regulatory proteins in growth, all mutant strains were grown planktonically and their growth rates compared to *P. aeruginosa* PAO1 wild type. No difference in growth rates were observed indicating that inactivation of these genes did no affect fitness.

The role of regulatory proteins in initial attachment and early biofilm developmental stages was assessed using a microtiter plate assay in which *P. aeruginosa* wild type and mutant cells were grown for 2-12 hours, after which time the attached biomass was quantified as using crystal violet.

Inactivation of bfiS encoding for biofilm-specific regulatory protein (Table 1) did not affect initial attachment to polystyrene compared to wild type biofilms as revealed by the crystal violet microtiter plate assay. These findings were confirmed by microscopy (not shown). A *ΔgacS* mutant showed reduced initial attachment, consistent with previous findings by Parkins and colleagues (Parkins *et al.*, 2001). Inactivation of *plkR* (see Table 1) resulted in significantly increased attachment. Complementation of *ΔplkR* restored the wild type attachment phenotype. Attachment of *ΔsagS* mutants was increased within the first 2-4 hours of attachment as determined by CV staining and microscopy (not shown). Inactivation of *sagS* resulted in significantly enhanced attachment, with *ΔsagS* attaching twice as efficiently as PAO1 within the first two hours following initial attachment (Fig. 1A). The findings are consistent with previous reports by Goodman et al. demonstrating increased attachment capabilities for this mutant (Goodman *et al.*, 2004). However, increased attachment by *ΔsagS* was only temporal, and the difference in attachment diminished with increasing attachment times. By 8 hr, no difference in attachment was observed between PAO1 and *ΔsagS.* Overexpression of *sagS* did not affect attachment. Complementation of the *ΔsagS* mutant restored attachment to wild type levels (not shown). Growth curves in liquid media suggested that the increase in attachment was not a result of a general increase in growth rate.

Role of biofilm-specific regulatory proteins in biofilm development.

As differential and sequential phosphorylation of regulatory proteins was detected over the course of *P. aeruginosa* biofilm development, we asked whether inactivation of these regulatory proteins would alter or affect the stage-specific progression of biofilm formation. Since the protein BfiS was found to be phosphorylated following 82 hr of biofilm growth (Table 1), a *bfiS* mutant was chosen and allowed to form biofilms for 144 hr in flow cells to test for biofilm formation defects.

Under the conditions tested, wild type *P. aeruginosa* biofilms reached maturity following 144 hr of growth as characterized by biofilms being composed of large microcolonies exceeding 100 μm in diameter. In contrast, a *bfiS* mutant biofilm lacked clusters and microcolonies after 144 hr of growth (Fig. 3B) and were only composed of a thin layer of cells at the substratum with an average height of 0.5 and 1.4 μm, respectively (Table 3). Furthermore, the mutant biofilm differed significantly from wild type biofilms with respect to biomass, surface coverage, and roughness coefficient. Complementation of the *bfiS* mutant restored biofilm formation to wild type levels while overexpression increased the biofilm biomass compared to wild type biofilms grown for the same amount of time (Fig. 3, Table 3). These results allowed us to firmly conclude that the mutant biofilm phenotype is caused by a defect in BfiS.

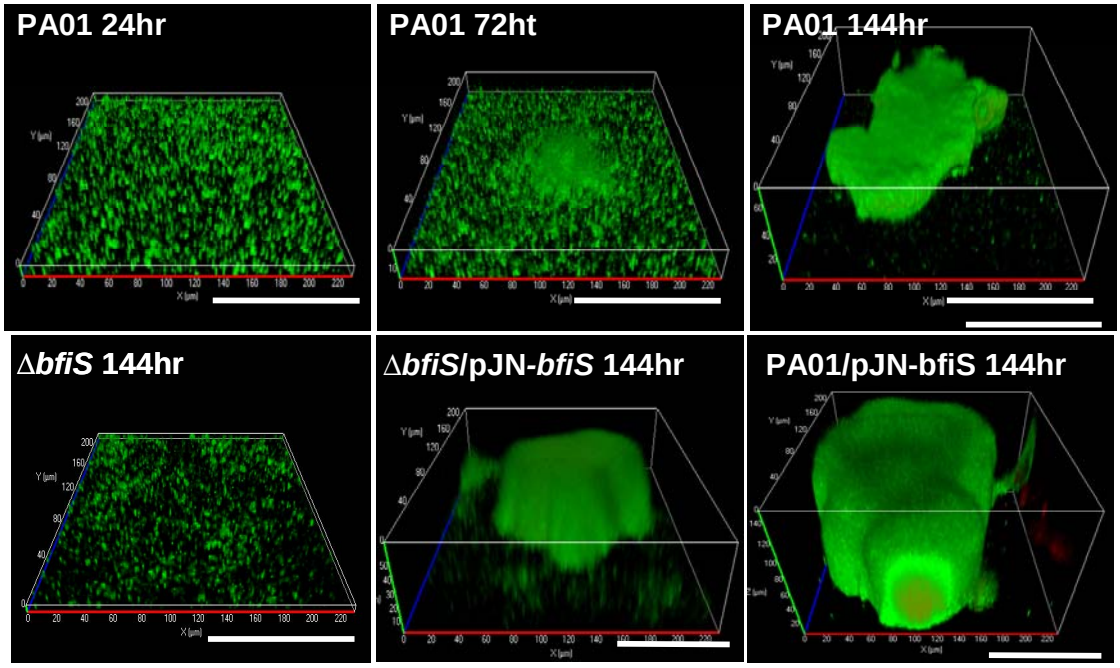

Fig. 2. *P. aeruginosa* mutant strain inactivated in or overexpressing *bfiS*, grown for 144 hours, were visualized by CSLM and compared to wild type PAO1 biofilms at various stages of development. White bar = 100µm.

To exclude the possibility that the *ΔbfiS* mutant biofilm may have disaggregated prematurely, the formation of mutant biofilms was monitored daily by confocal microscopy over a period of 144 hr. No difference in biofilm biomass and architecture was observed when *ΔbfiS* biofilms ranging in age between 24 and 144 hr were compared (not shown), indicating that the observed mutant biofilm architecture was not due to premature biomass disaggregation. The findings clearly indicate that inactivation of this novel regulatory protein did not cause biofilm disaggregation. Instead, our findings suggested that the mutant biofilm was incapable of progressing from the initial attachment stage to more mature biofilm stages.

Table 3. COMSTAT analysis[a] of *P. aeruginosa* wild type and mutant biofilm structure.

Strains	Total biomass ($\mu m^3/\mu m^2$)	Substratum coverage (%)	Average thickness (μm)	Maximum thickness (μm)	Roughness coefficient
***P. aeruginosa* PAO1**					
PAO1 (24 hrs)	0.95 (±0.39) [1, 2]	12.11 (±5.08) [1, 2]	0.78 (±0.38) [1, 2]	7.8 (±1.14) [1, 2]	1.69 (±0.12) [1, 2]
PAO1 (72 hrs)	3.27 (±1.29) [2]	32.43 (±8.31)	3.02 (±1.52)	18.29 (±10.86)	1.20 (±0.31)
PAO1 (144 hrs)	7.99 (±3.63) [1]	44.44 (±22.58)	7.17 (±4.97)	35.63 (±26.73)	1.24 (±0.37)
Mutant biofilms following 144 hours of growth					
ΔPA4197 (ΔbfiS)	1.01 (±0.44) [1,2]	17.19 (±6.42) [1,2]	0.51 (±0.53) [1,2]	6.55 (±3.94) [1,2]	1.78 (±0.19) 1, 2
ΔPA4197/pJN4197	9.48 (±2.92)	33.05 (±11.53)	8.22 (±2.85)	44.85 (±9.17)	1.44 (±0.20)
PAO1/pJN4197	30.12 (±15.92)	31.54 (±12.23)	31.03 (±17.31)	89.78 (±29.34)	1.12 (±0.42)

[a] Comstat analysis was carried out from biofilms grown in triplicate using at least 6 images per replicate.
[1] Significantly different from PAO1 72 hr biofilm values ($p \leq 0.05$) as determined by ANOVA.
[2] Significantly different from PAO1 144 hr biofilm values ($p \leq 0.05$) as determined by ANOVA

Table 4. COMSTAT analysis[a] of *P. aeruginosa* wild type and complemented mutant biofilm structure following removal of arabinose and thus, lack of expression of *bfiS*.

Strains	Time (hr)b	Total biomass (μm3/μm2)	Substratum coverage (%)	Average thickness (μm)	Maximum thickness (μm)	Roughness coefficient (dimensionless)
PAO1/pJN105	0	16.55 (±11.18)	25.42 (±12.98)	17.44 (±11.48)	75.51 (±16.60)	1.30 (±0.31)
	72	25.58 (±18.21)	32.28 (±21.77)	27.77 (±23.30)	77.22 (± 32.35)	1.05 (±0.49)
	144	39.69 (±18.73)	58.99 (±19.68)	38.06 (±19.17)	76.56 (±33.66)	0.71 (±0.43)
ΔbfiS/pJN-*bfiS*	0	18.53 (±14.12)	20.22 (±10.57)	20.45 (±16.46)	73.72 (±19.89)	1.36 (±0.39)
	72	2.94 (±2.31) *	12.63 (±7.25) *	2.81 (±2.51) *	16.61 (±7.89) *	1.56 (±0.32) *
	144	2.60 (±3.77) *	12.50 (±12.23) *	2.25 (±3.43) *	16.47 (±7.92) *	1.69 (±0.30) *

[a] Comstat analysis was carried out from biofilms grown in triplicate using at least 6 images per replicate.
[b] Time 0 = 144-hr-old biofilms.
* Value significantly different ($p \leq 0.05$) from PAO1 biofilm on corresponding day as determined by ANOVA.

Loss of expression of the biofilm-specific regulatory protein BfiS in mature biofilms results in the disaggregation of biofilms and biofilm biomass loss.

Our observations indicated that BfiS is essential in the stage-specific development of *P. aeruginosa* biofilm formation. To determine whether these regulatory proteins are only essential during biofilm formation or are also necessary for the maintenance of established biofilms, we asked whether inactivation of these regulatory proteins in mature biofilms would affect biofilm architecture. Complemented mutant strains, harboring the respective regulator genes under the control of the arabinose-inducible P_{BAD} promoter, were allowed to grow for 144 hr in flow cells to maturity (Fig. 3 – 0 hr) in the presence of arabinose, after which time arabinose was removed from the growth medium to stop the transcricription of the respective genes. The resulting biofilm architecture was viewed over a period of 144 hr post arabinose removal using confocal microscopy. *P. aeruginosa* wild type harboring an empty pJN105 vector was used as control.

Loss of *bfiS* expression due to arabinose removal resulted in the collapse of the mutant biofilm architecture within three days. For *ΔbfiS* mutant biofilms, biofilm disaggregation was noticeable as early as 24 hr post arabinose removal. The collapse was apparent by significant reduction ($P < 0.05$) of biofilm variables including biofilm biomass and thickness, which further decreased upon continued incubation (Fig. 3, Table 4). Post 144 hr of arabinose removal, the biofilm architecture of the complemented mutants was similar to mutant biofilms lacking the respective regulatory gene (Figs. 2-3). In contrast, no reduction of the wild type biofilm architecture was observed (Fig. 3, Table 4). These findings indicated that the three novel regulators are not only essential for the stage-specific progression of *P. aeruginosa* biofilms but also in the maintenance of the biofilm structure.

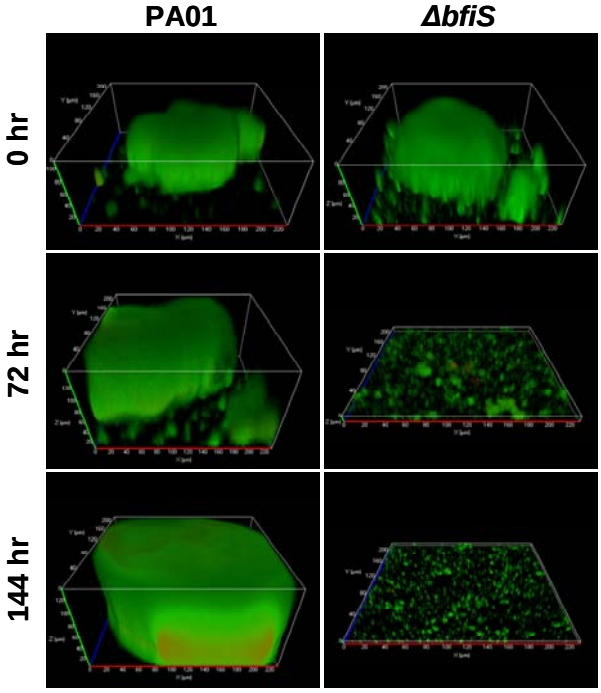

Fig. 3. Inactivation of *bfiS* expression in mature biofilms results in biofilm architectural collapse and biomass loss. *P. aeruginosa* mutants complemented with plasmid-borne copies of the *bfiS* gene placed under the regulation of the arabinose-inducible P_{BAD} were grown under continuous flow conditions in glutamate minimal medium (Sauer and Camper, 2001) in the presence of 0.1% arabinose for 144 hr after which time the biofilms were visualized by confocal microscopy (0 hr). Then, arabinose was eliminated from the growth medium and the biofilm architecture monitored post arabinose removal at the times indicated. PAO1 strain harboring the empty pJN105 vector was used as control. White bars = 100 μm, black bars = 25 μm.

Role of biofilm-specific regulatory proteins in protein phosphorylation.

Based on qualitative and quantitative analyses, BfiS mutant biofilm architecture appeared to be the result of arrested biofilm formation following initial attachment. Since each of these biofilm developmental stages is characterized by a unique phosphorylation pattern (Fig. 1, Table 1), we reasoned that if the bfiS mutant biofilm is indeed arrested in biofilm development, its

phosphoproteomes will correspond to the stage at which they are arrested. We, therefore, analyzed the phosphorylation patterns of *ΔbfiS* biofilms grown for 144 hr in comparison to *P. aeruginosa* wild type biofilms grown for 8, 24, 72, and 144 hr. The phosphoproteomes were analyzed using two approaches, (i) immunoblot analysis of whole biofilm cell extracts and (ii) LC-MS/MS analysis in conjunction with cICAT labeling following MOAC purification.

Based on overall similarity in phosphorylation events between planktonic and biofilm cells, the phosphorylation patterns of *ΔbfiS* biofilms demonstrated significantly greater similarity to the phosphoproteomes of planktonic cells than to mature, 144-hour-old *P. aeruginosa* wild type biofilms. The phosphoproteome of *ΔbfiS* biofilms as determined by LC-MS/MS was 74% similar (26% difference) to planktonic cells. This is in contrast to the phosphoproteome of 144-hr-old *P. aeruginosa* wild type biofilms, which was 62-65% different from that of planktonic cells. Furthermore, the bfiS mutant biofilm failed to exhibit phosphorylation events typically observed during normal biofilm development following 144 hr of growth. For instance, *ΔbfiS* biofilms lacked all phosphorylated proteins typically found in mature, 144-hr-old biofilms. Instead, *ΔbfiS* biofilms exhibited stage-specific phosphorylation events typically detected in 8-hr- and 24-hr-old wild type biofilms: the Ser/Thr phosphoproteome contained 15 out of 23 phosphorylated proteins and 2 out of 21 phosphorylated proteins that are specific for 8-hr- and 24-hr-old wild type biofilms, respectively (not shown).

Role of biofilm-specific regulatory proteins in protein production.

Since each biofilm developmental stage is not only characterized by unique Ser/Thr phosphoproteomes but also by unique protein production patterns as previously demonstrated using a proteomic approach (Sauer *et al.*, 2002; Southey-Pillig *et al.*, 2005), we reasoned that if the mutant biofilms are indeed arrested in biofilm development, their whole proteomes will also correspond to the stage at which they are arrested. To further confirm that *ΔbfiS* biofilms are arrested in biofilm development, protein production patterns of 144-hr-old mutant biofilms were analyzed by 2D/PAGE and compared to the 2D-patterns of *P. aeruginosa* wild type biofilms grown for 8, 24, 72 and 144 hr using 2D ImageMaster Platinum software and heuristic clustering. As shown in Figure 4, cluster analysis based on protein similarity confirmed our previous findings obtained by microscopic and phosphoproteome analyses of mutant biofilms. *ΔbfiS* biofilms were more similar to 24-hr-old wild type biofilms than to wild type biofilms at more mature stages. The two protein patterns were more than 80% similar.

Based on analyses of biofilm architecture, as well as of protein production and phosphorylation patterns, our findings indicate that *ΔbfiS* biofilms are arrested in the transition from reversible to the irreversible attachment stage (8hr to 24-hr-old biofilms, respectively).

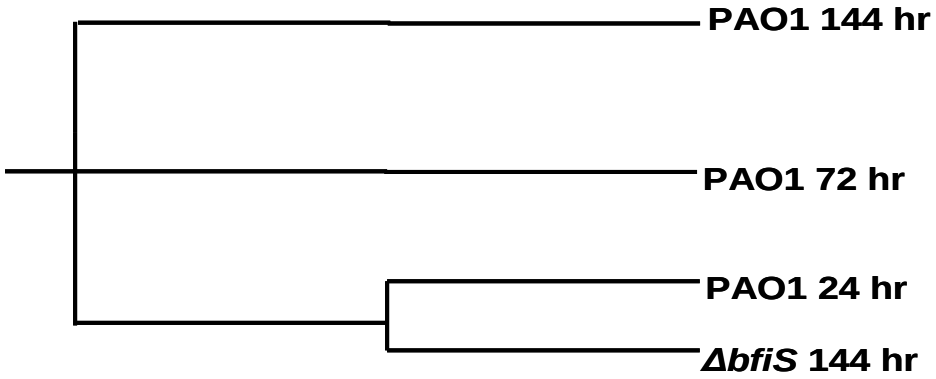

Fig. 4. Comparison of 2D/PAGE protein production patterns of wild type and bfiS mutant biofilms impaired in the developmental progression. Similarity of 2D-protein production patterns as determined by Heuristic clustering. Experiments were carried out in triplicate for each strain and/or biofilm age.

Identification of proteins that are differentially produced in mutants arrested in biofilm development

As indicated in Fig. 4, mutants arrested in biofilm development displayed altered phenotypes following 144 hrs of growth that were more comparable to early stage biofilms. The 2D/PAGE analysis further revealed that deletion of *bfiS* affected several biofilm specific proteins. Although we had not proposed to identify these proteins, we identified several proteins following tryptic digest by LC-MS-MS analysis.

By identifying proteins that were differentially produced in *ΔbfiS* biofilms compared to wild type biofilms (Tables 5-6), we were able to determine three sets of proteins that were affected by deletion of PA4197: Quorum sensing regulated proteins, *bfiS*-specific proteins (proteins that are only absent/present in *ΔbfiS* biofilms compared to wild type biofilms) and proteins that were specific to 24h biofilms.

Comparison of protein patterns of *ΔbfiS* mutant biofilm to protein patterns of the *ΔrhlI* and *ΔlasI* quorum sensing mutants comfirmed that *ΔbfiS* biofilm are lacking proteins that are controlled by the Rhl quorum sensing system (Table 5). We further confirmed that biofilms produced significantly less C4-acylated homoserine lactone (C4-HSL, Rhl quorum sensing signaling molecule) (not shown). These findings suggested a role of the Rhl quorum sensing system. We therefore tested whether addition of exogenous C4-HSL would restore the PA4197 biofilm phenotype. Interestingly, addition of exogenous C4-HSL to the growth medium of *ΔbfiS* biofilms did not restore the wild type phenotype (Fig. 5). In contrast, addition of exogenous C4-HSL to *ΔrhlI* biofilms resulted in the restoration of the wild type phenotype (Fig. 5). The findings

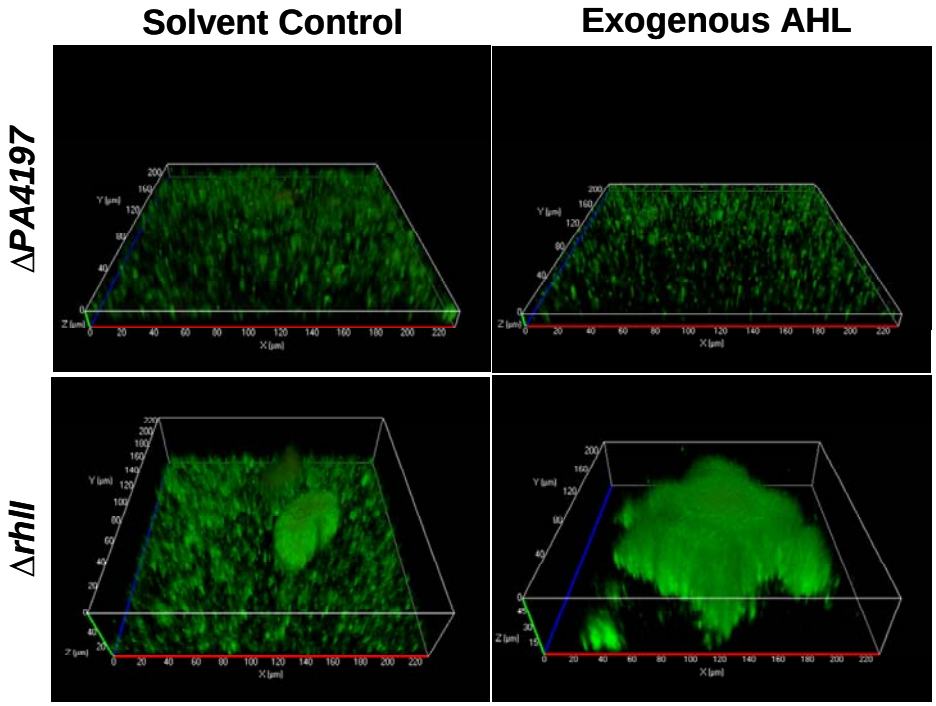

Figure 5. Biofilm architecture of *ΔrhlI* and *ΔPA4197* mutant biofilms in the absence and presence of exogenous acylated homoserine lactone. Biofilms were grown for 144 hrs and biofilms subsequently visualized by confocal microscopy.

indicate that the altered *ΔbfiS* biofilm phenotype is not dependent on quorum sensing.

Table 5. Identification of PA4197-specific and Quorum sensing regulated proteins that are differentially produced in *ΔPA4197* biofilms.

Strains						
PA01	***ΔbfiS***	***ΔlasI***	***ΔrhlI***			**Protein Identification**
Quorum Sensing regulated proteins						
v	-	v	-	PA2069	-	probable carbamoyl transferase
v	-	*v*	-	PA0051	*phzH*	potential phenazine-modifying enzyme
v	-	v	-	PA1900	*phzB2*	probable phenazine biosynthesis protein
v	-	*v*	-	*PA1546*	*hemN*	*oxygen-independent coproporphyrinogen III oxidase*
v	-	-	-	PA3329	-	hypothetical protein
v	-	v	-	PA4215	*phzF1*	probable phenazine biosynthesis protein
				PA1904	*phzF2*	probable phenazine biosynthesis protein
v	-	-	-	PA4498	-	probable metallopeptidase
v	-	-	-	PA1914	-	conserved hypothetical protein halovibrin
v	-	v	-	PA2300	*chiC*	chitinase
v	-	v	-	PA4217	*phzS*	flavin-containing monooxygenase probable FAD-dependent monooxygenase
v	-	-	-	PA1800	*tig*	trigger factor
-	v	v	v	PA2385	*pvdQ*	3-oxo-C12-homoserine lactone acylase PvdQ
-	v	v	v	PA0266	*gabT*	4-aminobutyrate aminotransferase gabA transaminase
v	-	v	v	PA3326	-	probable Clp-family ATP-dependent protease
***bfiS*-specific proteins**						
-	v	-	-	PA5016	aceF	dihydrolipoamide acetyltransferase
-	v	-	-	PA0963	aspRS	aspartyl-tRNA synthetase aspRS aspartate-tRNA ligase
-	v	-	-	PA4554	pilY1	type 4 fimbrial biogenesis protein PilY1
v	-	v	v	PA2249	bkdB	branched-chain alpha-keto acid dehydrogenase
v	-	v	v	PA1662	-	probable ClpA/B-type protease
v	-	v	v	PA4175	piv	protease IV "probable endoproteinase
v	-	v	v	PA5557	atpH	ATP synthase delta chain
-	v	-	-	PA1580	gltA	citrate synthase
-	-	-	-	PA5210	-	probable secretion pathway ATPase

Table 6. Identification of proteins that are unique to 24 hrs biofilms.

PA01			***ΔbfiS***					**Gene ID**		**Protein ID**
1D	**3D**	**6D**	**1D**	**3D**	**6D**	***ΔlasI***	***ΔrhlI***			
v	-	-	v	v	v	-	-	PA2491		probable oxidoreductase
-	*v*	*v*	-	-	-	*v*	*v*	PA2116		conserved hypothetical protein
v	-	-	v	v	v	v	v	PA3471		probable malic enzyme probable malate dehydrogenase
v	-	-	v	v	v	v	v	PA2023	*galU*	UTP--glucose-1-phosphate uridylyltransferase

Closer inspection of differentially expressed genes indicated that the overall changes in transcript levels following *bfiS* inactivation were unlike those detected in a *ΔrhlR* mutant. This is further supported by the finding that restoration of C4-HSL levels in *ΔbfiS* did not restore biofilm formation to wild type levels (Fig. 5). However, TTSS- and Rhl-regulated genes were expressed in *ΔbfiS* in a manner similar to those observed in *ΔgacA* and *ΔrsmYZ* (Heurlier *et al.*, 2004; Kay *et al.*, 2006; Pessi and Haas, 2001; Williams *et al.*, 2007) and opposite to a mutant inactivated in *rsmA*. We therefore explored a possible link between the novel TCS BfiSR and the LadS/RetS/GacAS/RsmA/*rsmYZ* multi-component system. This was done by analyzing the expression of genes central to this regulatory network, in particular *rsmA*, *rsmY* and *rsmZ*.

Under planktonic growth conditions, no difference in *rsmAYZ* gene expression was detected when transcript levels of PAO1/PA14 and Δ*bfiS* were compared. However, while *rsmA*, *rsmY*, and *rsmZ* abundance decreased up to 11-fold during wild type biofilm formation, deletion of *bfiS* coincided with unchanged *rsmA* and elevated *rsmYZ* levels in biofilm cells compared to the free-swimming counterparts (Fig. 6), indicating that BfiS-dependent modulation of *rsmYZ* are surface contact dependent. Interestingly, decreased transcript levels of *rsmA*, *rsmY*, and *rsmZ* were detectable as soon as 24 hr post initial attachment by *P. aeruginosa* wild type.

The findings suggested that once attached, *P. aeruginosa* wild type biofilm formation coincides with an overall decrease in the expression of *rsmA*, *rsmY* and *rsmZ*, a response absent in *ΔbfiS* biofilms.

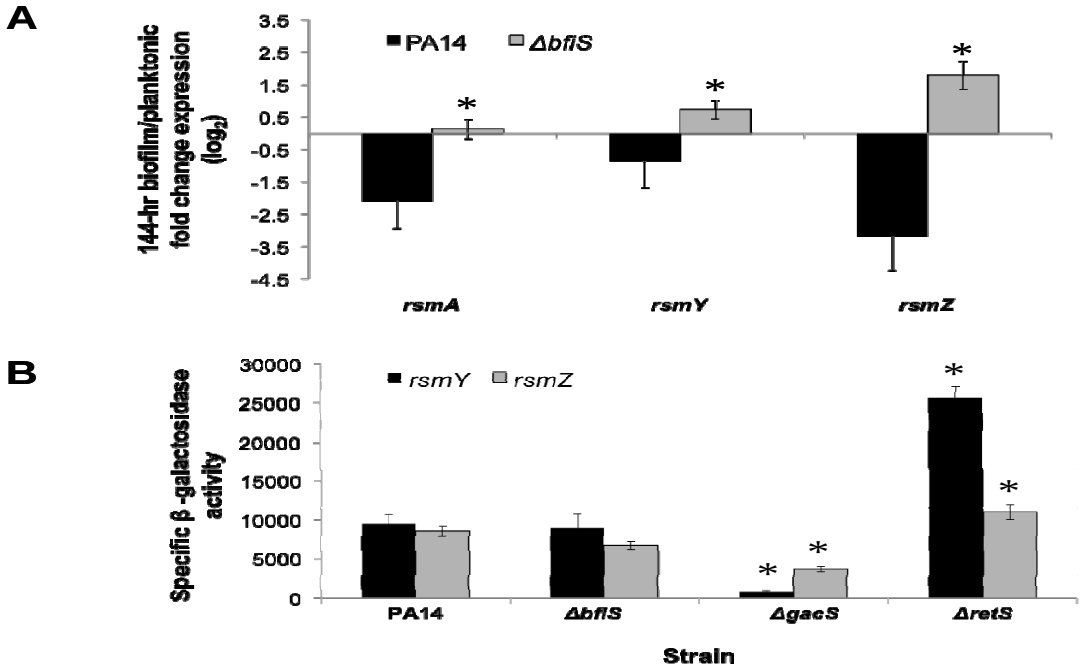

Fig. 6. Expression and transcript abundance levels of *rsmAYZ* in *P. aeruginosa* wild type and selected mutant strains. (A) Fold change in *rsmAYZ* levels in *P. aeruginosa* 144-hr-old wild type and *ΔbfiS* biofilms as compared to planktonic growth conditions were assessed by qRT-PCR analysis. (B) Transcriptional reporter fusion assay for *rsmY* and *rsmZ* expression in *P. aeruginosa* wild type, *ΔbfiS*, *ΔretS*, and *ΔgacS* 144-hr-old biofilms.

To determine whether differences in *rsmYZ* levels observed during wild type and *ΔbfiS* biofilm formation (Fig. 6A) are due to reduced transcription of *rsmYZ*, we made use of *rsmY* and *rsmZ* chromosomal transcriptional fusions and determined β-galactosidase activity in planktonic, 24- and 144-hr-old biofilm cells. Transcriptional fusion data showed no difference in *rsmY* and

rsmZ expression between *ΔbfiS* and its isogenic parental strain under planktonic (not shown) or biofilm growth conditions (Fig. 6B). Overall, *rsmY*- and *rsmZ-lacZ* reporter activity increased by 19- and 139-fold, respectively in *ΔbfiS* between 24 and 144 hr of biofilm growth (Fig. 7). Changes in RNA levels over the same period of time were comparable (Fig. 7). For wild type biofilms, transcription of *rsmY* and *rsmZ* changed 2.1- and 125.4-fold, respectively; while RNA levels only increased by 4.5- and 3.3-fold (Fig. 7). Thus, while *rsmYZ* transcription levels are similar in *ΔbfiS* and wild type regardless of growth conditions, *rsmYZ* abundance, in particular that of *rsmZ*, differs significantly between *ΔbfiS* and the wild type under biofilm growth conditions.

We next asked whether impaired biofilm formation due to *bfiS* inactivation is due to increased *rsmYZ* levels by assessing biofilm development by Δ*retS* and Δ*gacS*, strains previously shown to exhibit altered *rsmYZ* levels. Both *ΔbfiS* and *ΔretS* are characterized by increased *rsmYZ* levels as compared to wild type (Fig. 6A, (Goodman *et al.*, 2004)), however, they differ with respect to *rsmYZ* transcription (Fig. 6B). Moreover, in contrast to *ΔbfiS*, a *ΔretS* mutant hyperattaches to polystyrene and overexpresses genes required for Psl and Pel polysaccharide biosynthesis (Goodman *et al.*, 2004; Petrova and Sauer, 2009). The biofilm architecture of *ΔretS* was indistinguishable from a *ΔbfiS* (or *ΔbfiR*) mutant (Fig. 8), indicating that *rsmYZ* levels play an essential role in biofilm development. It is of interest to note that a *ΔgacS* mutant, characterized by decreased *rsmYZ* levels and decreased *rsmYZ* transcription under planktonic and biofilm (Fig. 6B) conditions, formed biofilms comparable to the wild type.

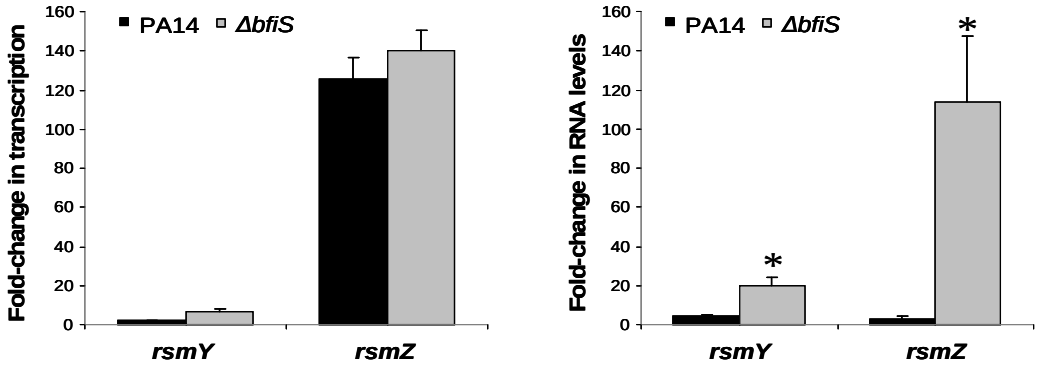

Fig. 7. Fold change in transcription and RNA levels of *rsmYZ* in *P. aeruginosa* wild type and *ΔbfiS* post attachment (24 hrs) upon continued biofilm growth (144hrs), as revealed by ß-galactosidase reporter assays and qRT-PCR, respectively. *Significantly different from respective wild type values ($p < 0.05$).

To determine which regulatory small RNA, *rsmZ* or *rsmY*, is responsible for biofilm development being arrested in a manner similar to inactivation of *bfiS* (or *bfiR*), strains overexpressing the sRNAs were tested. When placed in a PA14 background (PA14/pJN-*rsmY*), overexpression of *rsmY* resulted in biofilms that were overall similar in architecture to wild type biofilms, being composed of large microcolonies exceeding 100 µm in diameter (Fig. 8). However, quantitative analysis of the biofilm architecture by COMSTAT revealed a 50% reduction in the overall biomass of PA14/pJN-*rsmY* biofilms compared to PA14 (not shown). In contrast, biofilms overexpressing *rsmZ* (PA14/pJN-*rsmZ*) lacked large microcolonies and instead were composed of a thin layer of cells at the substratum with an average height of 4.5 µm and the presence of smaller cellular aggregates which were less than 30 µm in diameter (Fig. 8). Qualitative and quantitative analyses confirmed biofilms formed by the *rsmZ*-overexpressing strain (PA14/pJN-*rsmZ*) to be comparable to *ΔbfiS* and *ΔbfiR* biofilms, as well as to *ΔretS* biofilms (Fig. 8). Similar results were obtained when overexpression studies of *rsmY* and *rsmZ* were carried out in PAO1 (not shown).

These findings indicate that the arrest in biofilm development due to *bfiS* inactivation is mainly based on increased levels of *rsmZ*. Furthermore, the data indicate that *rsmY* and *rsmZ* do not appear to have redundant functions in biofilms.

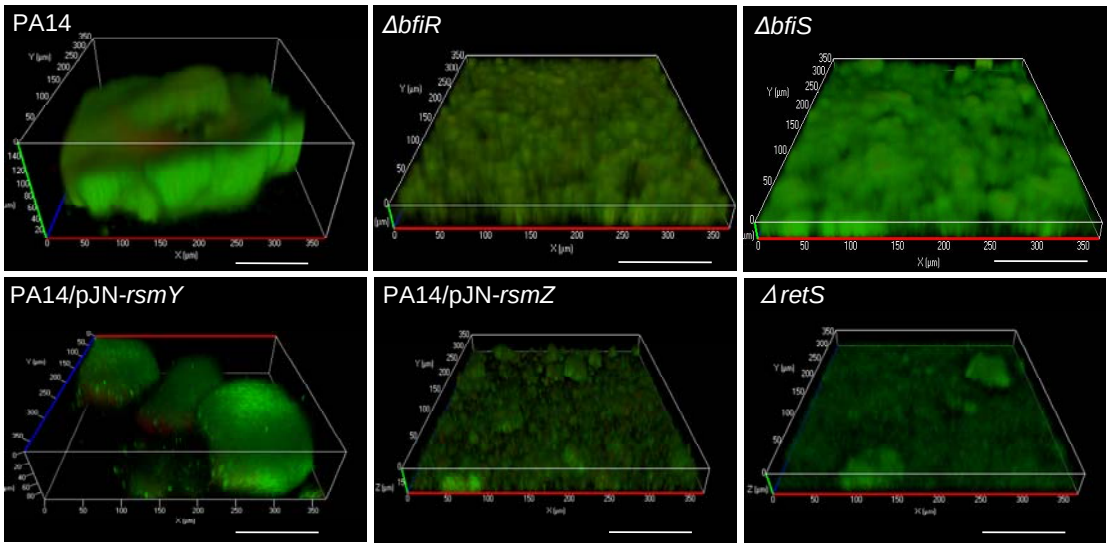

Fig. 8. **Biofilm architecture of *P. aeruginosa* PA14 and mutants inactivated in or overexpressing *bfiR, bfiS, retS, rsmY, and rsmZ.*** Biofilms were grown for 144 hrs, stained with the LIVE/DEAD *Bac*Light viability stain (Invitrogen Corp.), and visualized by CSLM. White bar = 100μm.

BfiSR regulates biofilm development through ribonuclease G (CafA).

We next addressed the question of how BfiSR regulates the degradation of small RNAs. We hypothesized that this is accomplished indirectly through the regulation of genes/proteins involved in post-translational/transcriptional modifications (see Table 2). To determine the direct regulatory targets of BfiSR, chromatin immunoprecipitation (ChIP) analysis was consequently employed to identify the DNA binding sites of BfiR, the cognate response regulator of BfiS. Under the conditions tested, we identified sequences located directly upstream of PA4477, a gene encoding the ribonuclease G CafA (Fig. 9A). CafA has been implicated in *E. coli* in 16S RNA maturation and rapid decay of mRNA (Kaga *et al.*, 2002; Tock *et al.*, 2000; Umitsuki *et al.*, 2001). The binding of BfiR to the sequence upstream of *cafA* was verified by electrophoretic mobility shift assays (Fig. 9B), with BfiR binding to the sequence present in all identified *cafA* ChIP clones (-75 to +100 relative to translational start site, $P_{cafA}1$), as well as to 75 bp directly upstream of the translational start site ($P_{cafA}2$). In addition, *cafA* expression was found to be BfiS-dependent with deletion of *bfiS* resulting in a 5(±0.52)-fold reduction of *cafA* transcript abundance (not shown).

While *ΔcafA* displayed normal attachment to polystyrene using a microtiter plate assay (not shown), *ΔcafA* failed to develop biofilm architecture observed in mature wild type biofilms following 144 hr of growth and accumulated 10-fold less biomass over the same period of time (Fig. 9C). However, *ΔcafA* biofilms were similar to *ΔbfiS* in architecture, in that both strains produced flat unstructured biofilms devoid of large microcolonies and complex differentiated structures. Further support for the role of CafA was obtained by the finding of increased *rsmA*, *rsmY*, and *rsmZ* levels in a *cafA* mutant biofilm (Fig. 9D). The changes in expression were comparable to those observed for *ΔbfiS* biofilms as compared to its isogenic parental strain PA14 (Fig. 9D). To confirm that the *ΔbfiS* biofilm phenotype was due to reduced *cafA* expression, we overexpressed *cafA* in a *ΔbfiS* mutant and analyzed the biofilm architecture. Overexpression of *cafA* in a *ΔbfiS* mutant restored the biofilm architecture to wild type levels (Fig. 9C). Importantly, *cafA* overexpression also resulted in 10-fold reduced *rsmZ* levels in *ΔbfiS* biofilms (not shown).

These findings confirmed BfiSR to be involved in coordinating biofilm formation through CafA.

To determine whether CafA is responsible for the sRNA turnover observed in *P. aeruginosa* wild type biofilms, *rsmA* and *rsmYZ* levels were determined in biofilms overexpressing *cafA* (PA14/pJN-*cafA*). *P. aeruginosa* harboring a blank vector (PA14/pJN105) was used as control. No differences in *rsmA* and *rsmY* levels were detected. However, *rsmZ* levels were found to be 3.4(±0.44)-fold reduced in PA14/pJN-*cafA* compared to PA14/pJN105. The finding indicates CafA, which was implicated in *E. coli* in wide-scale mRNA maturation and turnover processing (Tock *et al.*, 2000; Umitsuki *et al.*, 2001), to specifically target *rsmZ* under biofilm growth conditions in *P. aeruginosa*.

In conclusion, our data suggest that BfiSR plays an essential role in virulence and biofilm development by *P. aeruginosa* by triggering global changes in the production of genes/proteins associated with a wide array of functions including virulence, transport and secretion, QS, and post-translational/transcriptional modification. According to the results of this study, most if not all major effects of BfiSR can be ascribed to the combined action of *rsmY* and *rsmZ.* There are multiple levels of regulation to modulate sRNAs. The observation that BfiSR modulates *rsmYZ* RNA abundance with ribonuclease G affecting *rsmZ* adds an additional level of signaling potential and regulation to an already complex sRNA-modulating system. A more thorough understanding of how bacteria activate BfiSR to regulate sRNAs may be key for developing novel strategies to target biofilms.

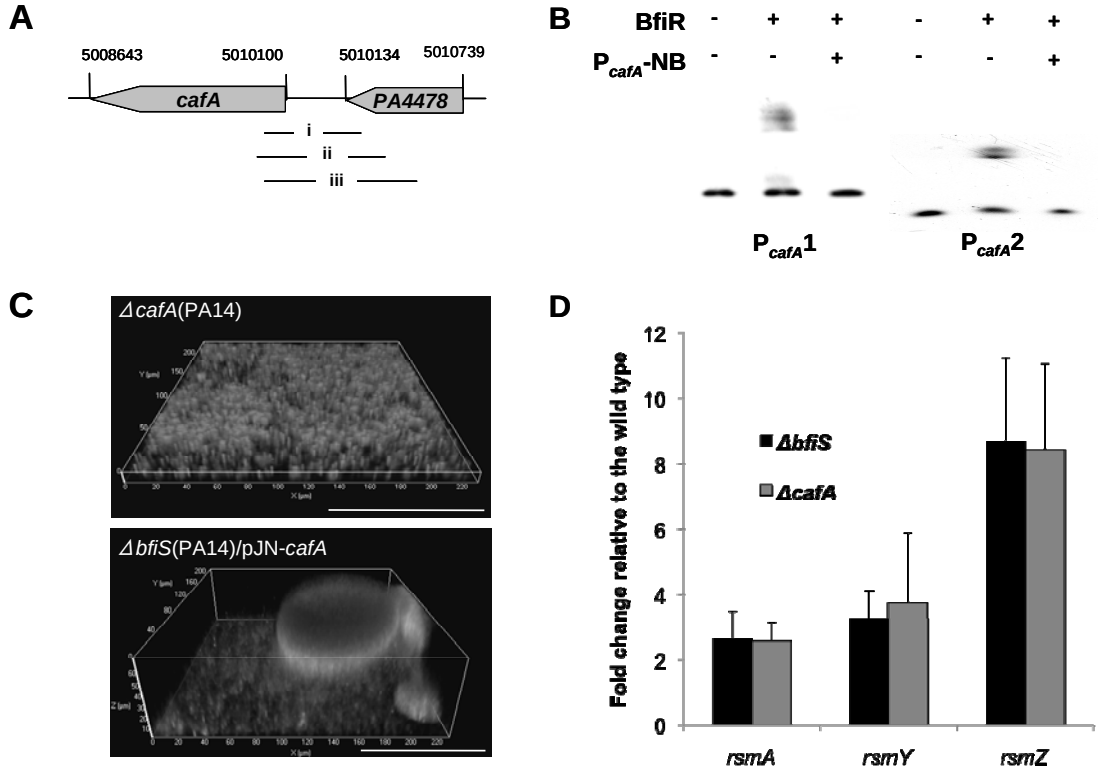

Fig. 9. BfiR binds to sequences upstream of *cafA*, with *cafA* expression modulating *rsmYZ* levels and being required for normal biofilm development by *P. aeruginosa.* (A) DNA binding sites upstream of *cafA* under biofilm growth conditions as identified by ChIP analysis. Lines labeled i-iii indicate location of three separate ChIP-library inserts: (i) 5009703-5010114; (ii) 5009701-5010182; (iii) 5009796-5010287. (B) Gel mobility shift analysis of BfiR-*cafA*. $P_{cafA}1$ (-75 to +100 bp relative to translational start site, representing the sequence present in all identified *cafA* ChIP clones) or $P_{cafA}2$ (-75 to 0 bp relative to translational start site) was incubated in the presence or absence of BfiR. Non-biotinylated $P_{cafA}1$ or $P_{cafA}2$ (P_{cafA}-NB) was used as specific competitor DNA. (C) Confocal images of 144-hr-old mutant biofilms inactivated in or overexpressing *cafA*. White bar = 100 μm. (D) *rsmAYZ* levels in *ΔbfiS* and *ΔcafA* biofilms compared to *P. aeruginosa* wild type biofilms, as assessed by qRT-PCR.

Inactivation of *sagS* encoding the two-component regulatory sensor hybrid leads to altered biofilm formation.

Since inactivation of *sagS* had only a temporary positive effect on attachment, with the difference in attachment diminishing upon continued surface exposure, we asked whether differential expression of this regulatory protein would alter or affect biofilm formation. We anticipated detecting no difference in *ΔsagS* biofilm formation as compared to PAO1. However, the mutant strain failed to develop the biofilm architecture typically observed in PAO1 biofilms following 144 hr under biofilm growth conditions (Fig. 10). Instead, *ΔsagS* formed only thin biofilms that lacked large cellular aggregates and microcolonies and were composed of ≥5-fold less biomass as compared to the wild type. Overall, *ΔsagS* biofilms most closely resembled those of PAO1 following 24 hr of growth. In contrast, overexpression of *sagS* resulted in biofilms that were similar in architecture to PAO1 biofilms, but displayed significantly increased biomass, as well as average and maximum thickness when grown for 144 hr (Fig. 10, Table 7). Daily monitoring of *ΔsagS* biofilms by confocal microscopy over a period of 144 hr indicated that the observed biofilm architecture was not due to premature disaggregation. Instead, *ΔsagS* biofilms failed to accumulate additional biomass typically seen in wild type biofilms following continued growth under flowing conditions (post 48 hr of growth, Fig. 10 (Petrova and Sauer, 2009; Sauer *et al.*, 2002)). The findings suggested *ΔsagS* biofilms to be arrested in biofilm development compared to the wild type. Similar results with respect to biofilm architecture were observed when *ΔsagS* mutants were grown in VBMM minimal medium (not shown).

Overexpression of *plkR* leads to biofilms displaying large voids and increased cellular motility.

plkR inactivation favored interactions with the surface during the early stages of attachment, while *plkR* overexpression had no effect on initial attachment over a period of 8 hr. Despite the hyperattachment phenotype of *ΔplkR*, biofilms formed by *ΔplkR* were indistinguishable from those of PAO1 (Fig. 10, Table 7). The finding suggests that PlkR does not play a role in biofilm development once *P. aeruginosa* has transitioned to the surface. In contrast, overexpression of *plkR* had a pronounced effect on biofilm formation, in that it resulted in significant reductions in biofilm biomass accumulation and biofilm thickness when compared to wild type biofilms (Fig. 10). Furthermore, large void areas devoid of bacterial cells were observed at the substratum (Fig. 10, Table 7) and cells within the substratum layer demonstrated increased cellular motility within biofilms (see supplement for movie) indicative of dispersion events (Sauer *et al.*, 2002).

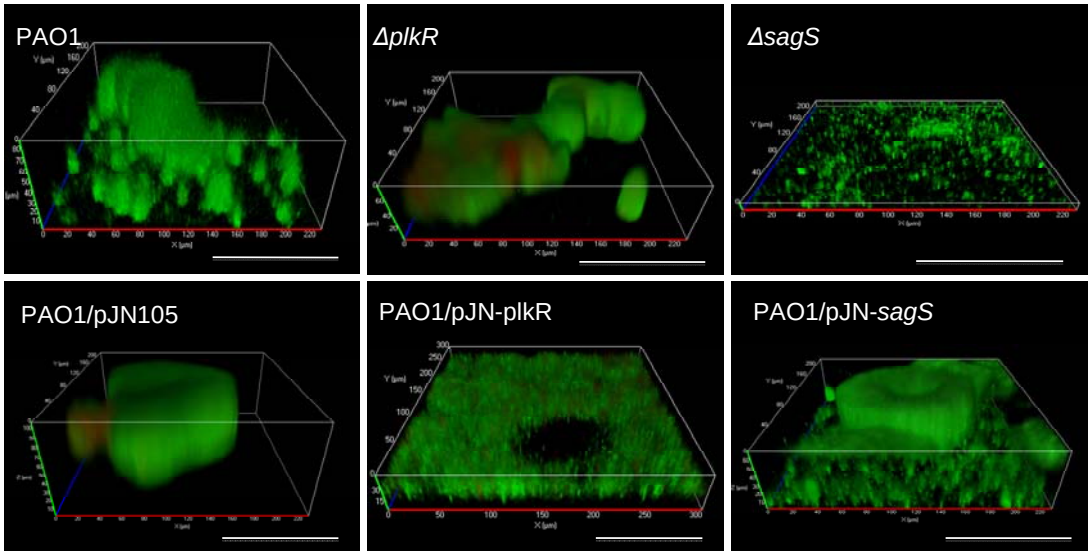

Fig. 10. Biofilm formation of *P. aeruginosa* wild type and mutant strains inactivated in or overexpressing *plkR* and *sagS*. White bar = 100 µm.

Table 7. COMSTAT analysis[a] of *P. aeruginosa* wild type and mutant biofilm structure.

Strains	Total biomass ($\mu m^3/\mu m^2$)	Substratum coverage (%)	Average thickness (μm)	Maximum thickness (μm)	Roughness coefficient (dimensionless)
***P. aeruginosa* PAO1**					
PAO1 24hr	0.95 (±0.39)*	12.11 (±5.08)*	0.78 (±0.38)*	7.8 (±1.14)*	1.69 (±0.12)*
PAO1 72hr	3.27 (±1.29)*	32.43 (±8.31)	3.02 (±1.52)*	18.29 (±10.86)*	1.20 (±0.31)
PAO1 144hr	7.99 (±3.63)	44.44 (±22.58)	7.17 (±4.97)	35.63 (±26.73)	1.24 (±0.37)
***P. aeruginosa* PAO1 isogenic mutants following 144 hr of growth**					
ΔplkR	8.90 (±5.57)	32.44 (±22.76)	7.38 (±4.63)	36.63 (±18.28)	1.18 (±0.51)
PAO1/pJN-PA0701 (*plkR*)	3.89 (±1.97)*	19.15 (±15.85)*	3.20 (±2.78)*	23.36 (±11.28)*	1.53 (±0.23)
ΔsagS	0.82 (±0.63)*	7.07 (±5.14)*	0.83 (±0.62)*	9.00 (±2.83)*	1.74 (±0.20)*
PAO1/pJN-PA2824 (*sagS*)	14.04 (±9.98)*	25.64 (±11.26)	16.97 (±14.51)*	55.49 (±28.84)	1.09 (±0.37)
***ΔbfiS*/pJN-PA2824**	2.10 (±2.54)*	8.12 (±6.98)*	1.89 (±2.48)*	27.71 (±16.19)	1.82 (±0.15)
***ΔsagS*/pJN-PA4197 (*bfiS*)**	1.33 (±1.59) *	4.79 (±2.00)*	1.24 (±1.78)*	27.33 (±13.08)	1.89 (±0.06)
***ΔsagS*/pJN-PA4196 (*bfiR*)**	27.76 (±14.60)*	28.11 (±12.21)	29.40 (±15.79)*	92.33 (±24.98)*	1.12 (±0.35)

[a] COMSTAT analysis was carried out from biofilms grown in triplicate using at least 6 images per replicate.

* Significantly different from 144-hr-old PAO1; $p \leq 0.05$ as determined by ANOVA and SigmaStat.

Table 8. COMSTAT analysis[a] of *P. aeruginosa* wild type and complemented mutant biofilm structure following removal of arabinose and thus, lack of expression of *sagS* and *plkR*, respectively.

Strains	Time (hr)[b]	Arabinose[c]	Total biomass ($\mu m^3/\mu m^2$)	Substratum coverage (%)	Average thickness (μm)	Maximum thickness (μm)	Roughness coefficient (dimensionless)
PAO1/pJN105	0		11.92 (±9.28)	28.85 (±13.29)	11.97 (±11.48)	37.17 (±26.78)	1.25 (±0.28)
	72	-	31.66 (±10.56)	29.37 (±11.36)	30.99 (±10.58)	66.33 (± 13.90)	1.22 (±0.30)
	144	-	36.92 (±18.00)	45.01 (±19.18)	37.91 (±18.76)	111.33 (±19.06)	0.92 (±0.44)
ΔsagS*/pJN-PA2824 *(sagS)	0		11.89 (±8.48)	25.34 (±11.36)	11.90 (±8.99)	47.67 (±29.96)	1.38 (±0.23)
	72	-	1.88 (±0.84)*	16.28 (±9.72)*	1.83 (±0.99)*	14.50 (±12.00)*	1.55 (±0.30)
	144	-	1.40 (±0.84)*	15.55 (±7.77)*	1.21 (±0.85)*	6.33 (±2.53)*	1.61 (±0.20)*
PAO1/pJN-PA0701 (*plkR*)	0		0.95 (±0.62)*	12.01 (±7.78)*	0.77 (±0.55)*	7.22 (±3.60)*	1.71 (±0.18)*
	72	-	11.54 (±11.47)*	16.02 (±10.99)*	11.19 (± 10.42)*	56.36 (±17.39)	1.60 (±0.29)*
	144	-	27.14 (±12.86)	26.64 (±7.77)*	28.19 (±13.20)	94.46 (±17.77)	1.25 (±0.31)
	72	+	1.82 (±1.16)*	16.65 (±9.10)*	1.69 (±1.16)*	10.36 (±6.05)*	1.58 (±0.23)*
	144	+	1.76 (±0.95)*	16.96 (±11.60)*	1.64 (±0.84)*	9.82 (±3.16)*	1.57 (±0.28)*

[a] COMSTAT analysis was carried out from biofilms grown in triplicate using at least 6 images per replicate.
[b] Time 0 = 144-hr-old biofilms, time corresponds to the time of media switch.
[c] (-) Samples switched to medium without arabinose following 144 hr of growth; (+) 0.1% arabinose retained in the medium following 144 hr of growth.
* Significantly different from PAO1/pJN105 at respective time point; $p \leq 0.05$ as determined by ANOVA and SigmaStat.

Expression of *plkR* is restricted to non-surface-attached growth and leads to biofilm dispersion.

The findings of PlkR reducing attachment but promoting biofilm disaggregation indicated a role of PlkR in maintaining or promoting a free-swimming lifestyle. We therefore asked whether *plkR* expression in biofilms promotes dispersion and/or return to the planktonic mode of growth by testing whether induction of *plkR* expression from an arabinose-inducible promoter in mature biofilms triggers dispersion events. Under the conditions tested, no dispersion events were detectable for the control strain harboring the blank vector (PAO1/pJN105) or for PAO1/pJN-PA0701 biofilms when switched to medium without arabinose. Induction of *plkR* expression, however, resulted in biofilm dispersion within 90 min following exposure of PAO1/ pJN-PA0701 biofilms to arabinose It is of interest to note that the pattern of dispersion triggered by *plkR* induction was unlike that previously observed for nutrient-induced dispersion.

Since induction of *plkR* expression in biofilms triggered dispersion events, we asked whether *plkR* expression promotes dispersion events or instead, whether expression of *plkR* is restricted to dispersed/free-swimming cells. We therefore induced dispersion conditions by exposing biofilms to sudden changes in environmental nutrient concentrations using excess glutamate (Morgan *et al.*, 2006; Sauer *et al.*, 2004). Resulting dispersed cells, biofilm cells remaining following dispersion, and uninduced biofilms were tested for *plkR* expression by qRT-PCR. *plkR* expression was not detected in biofilms prior to or post nutrient-induced dispersion. Furthermore, biofilms that had transitioned to the natural dispersion stage following a prolonged period of growth (beyond 144 hr in our system, see (Sauer *et al.*, 2002)) did not express *plkR* at detectable levels). Instead, *plkR* transcripts were detectable only in cells grown planktonically and cells that had dispersed from the biofilm, suggesting that *plkR* expression is specific to non-surface-attached cells.

PlkR and SagS regulate transitions through biofilm developmental stages in a reversible manner.

Our observations indicated that SagS (PA2824) was essential for the stage-specific development of *P. aeruginosa* biofilm formation, as *ΔsagS* was arrested in biofilm formation while *sagS* overexpression had no effect. In contrast, PlkR (PA0701) was found to be involved in the transition of biofilms to the dispersion stage as *plkR* inactivation had no effect on biofilm formation, while expression of *plkR* in biofilms coincided with the formation of large voids indicative of biofilm dispersal (Figs. 10). We therefore asked whether inactivation of these regulatory proteins in mature biofilms would affect biofilm architecture in a manner comparable to the effects on biofilm formation observed in *ΔplkR* and *ΔsagS.* We thus made use of complemented mutant strains which harbored the respective regulator genes under the control of the arabinose-inducible P_{BAD} promoter. In the case of *ΔsagS*, this allowed mutant biofilms to form wild type like biofilms within 144 hr of growth in the presence of arabinose (Fig. 11), after which time arabinose was removed from the growth medium to stop the transcription of the respective genes. The resulting biofilm architecture was viewed over a period of 144 hr post arabinose removal using confocal microscopy. *P. aeruginosa* wild type harboring an empty pJN105 vector was used as a control.

Loss of *sagS* expression due to arabinose removal resulted in the collapse of the mutant biofilm architecture within three days (Fig. 11, Table 8). While similar architectural collapse was observed following inactivation of *bfiS*, *bfmR*, and *mifR* expression (Petrova and Sauer, 2009), the collapse observed following *sagS* inactivation was distinct, as *sagS* inactivation coincided with biofilm dispersion as evidenced by the formation of large voids (Fig. 11) and increased motility of cells that remained attached at the surface (not shown). Large voids indicative of dispersion events were detectable as soon as 1 day following arabinose removal (not shown) and were visible for 6 days (Fig. 11). The collapse was apparent by significant reduction of biofilm variables including biofilm biomass and thickness, which further decreased upon continued

incubation. Under the conditions tested, no reduction of the wild type biofilm architecture was observed (Fig. 11). It is of interest to note that the *ΔsagS*/pJN-PA2824 biofilm architecture following *sagS* inactivation was distinct from *ΔsagS* biofilms (see Figs. 10-11), indicating the possibility of SagS playing a dual role in the maintenance of the free-swimming mode of growth and biofilm formation.

Post 144 hr of arabinose removal, the biofilm architecture of PAO1/pJN-PA0701 was similar to *ΔplkR*, as well as wild type biofilms, which do not express *plkR* (Figs. 10-11), indicating that removal of arabinose and thus, down-expression of *plkR* rescued biofilm formation by PAO1/pJN-PA0701 (Fig. 11, Table 8). It is of interest to note that continued induction of *plkR* for 144 hr in *ΔplkR*/pJN-PA0701 or PAO1/pJN-PA0701 biofilms by the addition of arabinose to the growth medium resulted in biofilms displaying dispersion events as indicated by the presence of large voids (not shown).

These findings indicate that the effect of the novel planktonic regulators SagS and PlkR is reversible with respect to biofilm architecture and biofilm development and that both are essential in the modulation of the free-swimming mode of growth by regulating the triggering dispersion events.

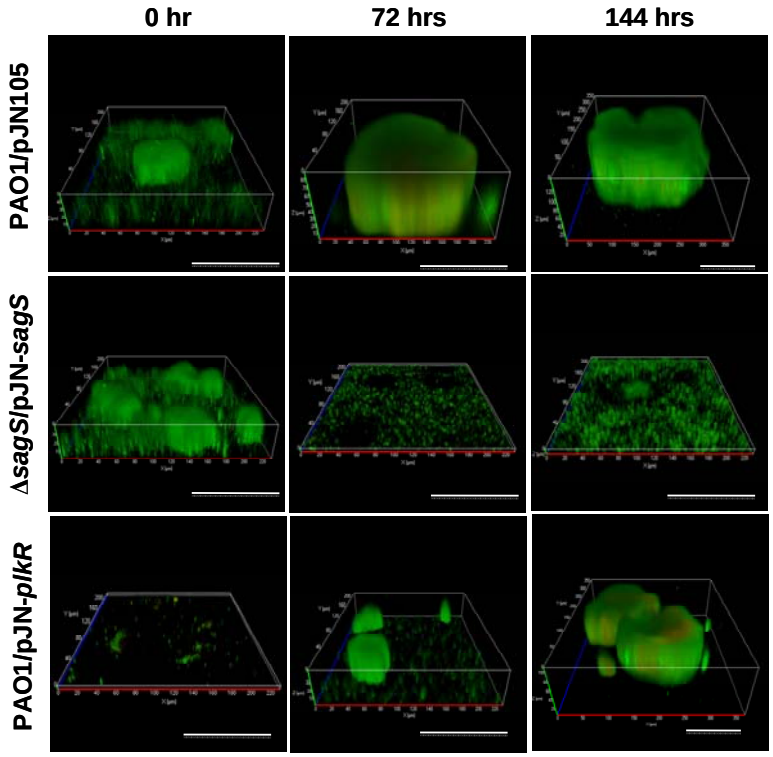

Fig. 11. Inactivation of *sagS* (PA2824) expression in mature biofilms results in biofilm architectural collapse and biofilm dispersion, while inactivation of *plkR* (PA0701) expression restores biofilm formation to wild type *P. aeruginosa* levels. Biofilms were stained with the LIVE/DEAD *Bac*Light viability stain (Invitrogen Corp.). White bar = 100 µm.

Inactivation of *plkR* coincides with decreased motility but increased adhesiveness and cyclic di-GMP levels.

To begin elucidating the mechanisms by which PlkR and SagS affect transitioning between the motile and the sessile states, mutants inactivated in and overexpressing *plkR* and *sagS* were tested for properties known to affect initial attachment including cellular motility, adhesiveness, and cyclic di-GMP levels.

No difference with respect to twitching, swimming, and swarming were detected for strains inactivated in or overexpressing *sagS* (not shown). Inactivation of *plkR*, however, did result in reduced swarming and swimming motility.

Mutant strains demonstrating increased attachment, increased adhesiveness but reduced swarming motility have been previously linked to increased cyclic di-GMP levels (Barraud *et al.*, 2009; Simm *et al.*, 2004). We therefore determined cyclic di-GMP levels in *ΔplkR* and PAO1

grown planktonically to assess whether differences in adhesiveness, attachment, and motility coincide with differences in cyclic di-GMP levels. Compared to *P. aeruginosa* PAO1, *ΔplkR* was found to contain 340% (187.9 ± 9.7 pmol/mg) higher cyclic di-GMP. The increased cyclic di-GMP levels by *ΔplkR* were comparable to levels typically detected in *P. aeruginosa* biofilms (not shown, see also (Barraud *et al.*, 2009)). In contrast, cyclic di-GMP levels detected in *ΔsagS* cells grown planktonically were comparable to *P. aeruginosa* PAO1 levels. Overall, these results strongly suggest that inactivation of *plkR* coincides with an increase of intracellular cyclic di-GMP levels, which results in enhanced attachment, increased adhesiveness, and reduced swarming and swimming motility.

Biofilm formation and initial attachment have been demonstrated to be affected by extracellular polymeric substances (EPS) (see (Flemming *et al.*, 2007) for review). In particular, polysaccharides have been shown to play a role in promoting biofilm formation in diverse *P. aeruginosa* strains (Friedman and Kolter, 2004; Jackson *et al.*, 2004; Ryder *et al.*, 2007). We therefore tested the strains inactivated in and overexpressing *plkR* and *sagS* for any aberrations associated with these properties by analyzing the expression of *pslA* involved in Psl polysaccharide biosynthesis. No significant difference in *pslA* expression was noted for *ΔplkR* and PAO1/pJN-PA0701 regardless of growth conditions as compared to *P. aeruginosa* PAO1 Similar results were obtained for the strain overexpressing *sagS* However, qRT-PCR demonstrated increased *pslA* expression in *ΔsagS* under free-swimming growth conditions but not in biofilms Increased Psl production by *ΔsagS* in a planktonic-specific manner was confirmed by Psl dot blot analysis. Under the conditions tested, Psl abundance by *ΔsagS* was comparable to the Psl-overproducing strain WFPA801.

SagS has been previously implicated in the modulation of small regulatory RNAs (sRNA) via the transfer of a phosphoryl group specifically to the histidine phosphotransfer protein HptB, which is in turn capable of phosphorylating the response regulator PA3346 and RetS (Bordi *et al.*, 2010; Hsu *et al.*, 2008). The activity of both HptB and RetS has been shown to intersect at the GacA response regulator (Bordi *et al.*, 2010; Hsu *et al.*, 2008), which directly controls sRNAs to reciprocally regulate the expression of exopolysaccharide components of the *P. aeruginosa* biofilm matrix, attachment, and swarming motility (Bordi *et al.*, 2010; Brencic and Lory, 2009; Brencic *et al.*, 2009; Goodman *et al.*, 2004; Heurlier *et al.*, 2004). Analysis of expression of *rsmA*, *rsmY*, and *rsmZ*, which are central to this regulatory network, revealed no difference in *rsmAYZ* levels in *ΔplkR* and PAO1/pJN-PA0701 regardless of growth conditions (Fig. 12A-B). However, inactivation of *sagS* correlated with elevated *rsmA* levels and significantly increased *rsmYZ* levels as compared to the wild type under planktonic growth conditions (Fig. 12A). *rsmAYZ* levels were only 2-fold increased in *ΔsagS* biofilms (Fig. 12B). Under planktonic growth conditions, increased *rsmYZ* levels by *ΔsagS* correlated with increased *pslA* expression and Psl production.

We have previously demonstrated that inactivation of *bfiS*, encoding a two-component sensor, arrested biofilm formation at the transition to the irreversible attachment stage (Petrova and Sauer, 2009). As *ΔsagS* biofilms appeared to be arrested at a similar stage of biofilm development as *ΔbfiS*, and as both mutants were previously described to be indirectly involved in modulating sRNAs (Bordi *et al.*, 2010; Hsu *et al.*, 2008; Petrova and Sauer, 2009; Petrova and Sauer, 2010), *rsmAYZ* levels were analyzed in *ΔbfiS* as well. The analysis demonstrated that *rsmAYZ* levels were unaltered in *ΔbfiS* under planktonic growth conditions but significantly increased upon biofilm growth as compared to wild type (Fig. 12A-B). These findings indicated that SagS and BfiS modulate *rsmAYZ* levels differently depending on the modes of growth. Similar results were obtained for *pslA* expression or Psl production.

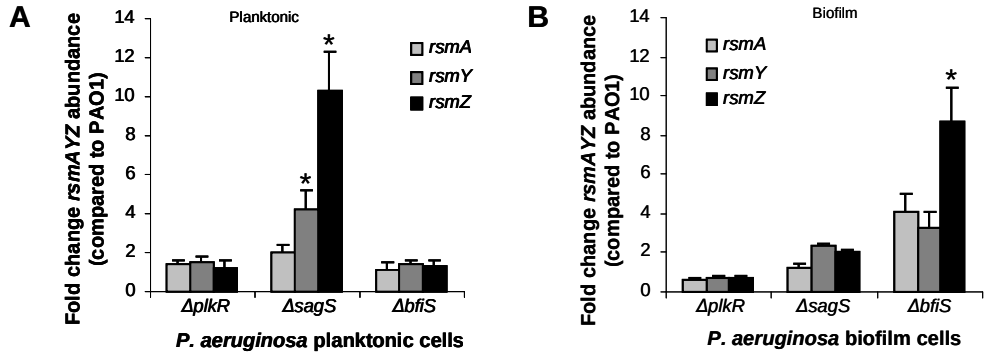

Fig. 12. Effect of inactivation and overexpression of *plkR, sagS,* and *bfiS* on *rsmAYZ* expression. *rsmAYZ* RNA levels by *P. aeruginosa* strains grown planktonically (A) and as biofilms (B) relative to wild type. Biofilms were grown for 144 hr under flowing conditions. All experiments were carried out in triplicate. *, significantly different from respective wild type grown under identical conditions/mode of growth ($p < 0.05$) as determined by Anova and Sigma Stat. Error bars indicate standard deviation.

Inactivation of *sagS* arrests biofilm formation at the transition to the irreversible attachment stage of biofilm development.

The findings of growth-dependent modulation of *rsmYZ* in *ΔsagS* compared to *ΔbfiS* indicated that SagS likely operates independently and/or upstream of BfiS/BfiR. However, both *ΔsagS* and *ΔbfiS* biofilms appeared to be the result of arrested biofilm formation (Figs. 2, 10). To determine at which biofilm developmental stage *ΔsagS* mutants are arrested, we made use of the finding that biofilm developmental stages are characterized by unique phosphorylation patterns (Fig. 1, (Petrova and Sauer, 2009)). A comparison of the phosphorylation patterns of *ΔsagS* biofilms grown for 144 hr to *P. aeruginosa* wild type biofilms grown for 8, 24, 72, and 144 hr indicated that *ΔsagS* biofilms failed to exhibit phosphorylation events typically observed during normal biofilm development following 144 hr of growth (Table 9). For instance, *ΔsagS* biofilms lacked all phosphorylated proteins typically found in mature, 144-hr-old biofilms, but instead exhibited stage-specific phosphorylation events typically detected in 8- and 24-hr-old wild type biofilms: the phosphoproteome contained 26 out of 38 phosphorylated proteins and 12 out of 33 phosphorylated proteins that are specific to 8- and 24-hr-old wild type biofilms, respectively (Table 9). In addition, *ΔsagS* biofilms did not exhibit BfmR or MifR phosphorylation. We have previously demonstrated that the novel TCS components BfmR and MifR are sequentially phosphorylated during biofilm development following 24 and 72 hr of growth, respectively (Petrova and Sauer, 2009). Furthermore, *ΔsagS* biofilms lacked evidence of BfiS phosphorylation which was previously demonstrated to be phosphorylated following 8 hr of biofilm growth (Petrova and Sauer, 2009), indicating that *ΔsagS* biofilms are probably arrested prior to *ΔbfiS,* at the transition from the planktonic to the initial attachment stage.

Comparison of the phosphorylation patterns of both *ΔsagS* and *ΔbfiS* mutant biofilms indicated that while both mutant biofilms exhibited stage-specific phosphorylation events typically detected in 8-hr- and 24-hr-old wild type biofilms, inactivation of *sagS* and *bfiS* affected distinct sets of phosphorylated proteins under biofilm growth conditions (Table 9).

Overexpression of *bfiR*, but not *bfiS*, restores biofilm formation by *ΔsagS* to wild type levels.

The phosphoproteome study indicated that while both gene products, BfiS and SagS, are required for *P. aeruginosa* biofilm development to occur, they do so via distinct but partly

overlapping phosphorylation and signaling pathways. We therefore reasoned that if SagS and BfiSR play distinct roles in biofilm formation, overexpression of *bfiS* or *bfiR* in *ΔsagS* would not result in restoration of wild type biofilm formation (and *vice versa*). However, if SagS and BfiSR have overlapping or sequential functions during biofilm development, overexpression of *bfiS* or *bfiR* is anticipated to restore biofilm formation by *ΔsagS* to wild type levels.

Overexpression of *sagS* in *ΔbfiS* had no effect on biofilm formation. Similarly, overexpression of *bfiS* encoding the sensor kinase in *ΔsagS* did not affect the overall biofilm architecture (Fig. 13). In contrast, overexpression in *ΔsagS* of *bfiR*, encoding the cognate response regulator of BfiS, resulted in restoration of the biofilm architecture (Fig. 13). Instead of forming thin biofilms lacking large cellular aggregates and microcolonies, *ΔsagS*/pJN-PA4196 (*bfiR*) biofilms were on average 30 µm thick, mostly due to the presence of large microcolonies. Overexpression of *bfiR* furthermore resulted in a 30- and 4-fold increases in biofilm biomass compared to *ΔsagS* and PAO1 biofilms of comparable age, respectively.

Table 9. Phosphorylation patterns of *ΔsagS* biofilms in comparison to *ΔbfiS* biofilms.

Biofilm developmental stage	**Detected phosphorylation events per indicated stage in wild type *P. aeruginosa* PAO1**	**Detected phosphorylation events in 144 hr mutant biofilms**		
		ΔbfiS	***ΔsagS***	**In common (*ΔbfiS*, *ΔsagS*)**
Planktonic	22	3	2	1
Motile-sessile switch*	8	6	6	5
Reversible attachment (8 hr)	38	25	26	18
Irreversible attachment (24 hr)	33	11	12	7
Maturation (144 hr)	39	0	0	0
Dispersion (216 hr)	19	0	0	0
Biofilm-specific**	7	6	7	6
Constitutive	26	26	26	26

*, present in both planktonic and initially-attached biofilm (8hrs and 24 hrs) cells (Petrova and Sauer, 2009).

**, present in all surface-attached cells regardless of biofilm stage or age (Petrova and Sauer, 2009).

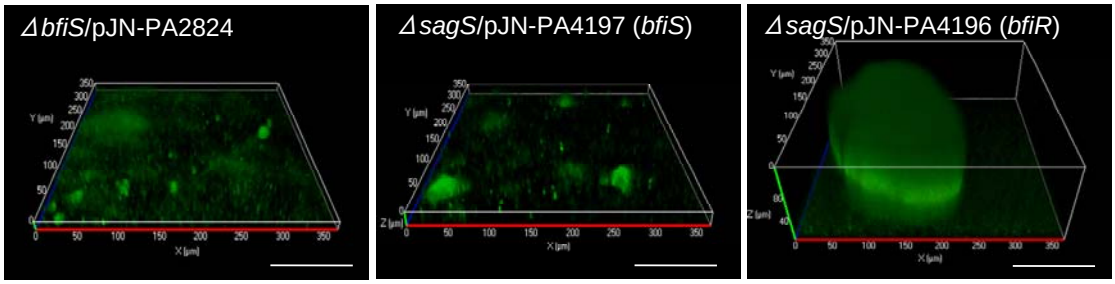

Fig. 13. Biofilm formation by *ΔsagS* is restored by overexpression of *bfiR*.

Taken together, our findings suggest that the stage-specific phosphorylation of BfiSR over the course of biofilm formation correlates with the specific regulatory role of this two-component regulatory systems essential for the progression of the developmental process. We furthermore demonstrate that planktonic-specific phosphorylation of regulatory proteins reflects their roles in free swimming bacterial cells, by modulating the motile-sessile/sessile-motile switch.

<u>Specific Aim 3.</u> Determine susceptibility of mutants impaired in the progression of biofilm developmental to antimicrobial treatment.

The focus of specific aim 3 was to investigate whether mutant biofilms impaired in the progression of biofilm development are more susceptible to antimicrobial agents than mature biofilms.

Independent of the antimicrobial agent tested, *P. aeruginosa* PAO1 biofilms were significantly more resistant as compared to planktonic cells. Furthermore, we noted a correlation between biofilm age and resistance. Mature biofilms (6 day old) were more resistant than younger biofilms (e.g. 1 day old). To further characterize the gradual decrease in resistance over the course of biofilm development by *P. aeruginosa*, we made use of a suite of mutants inactivated in plkR, sagS, and bfiS which are required for *P. aeruginosa* biofilm formation in a sequential manner. The regulatory proteins (SagS, BfiS, and PlkR) may form a signaling cascade (PlkR, SagS>BfiS) required for the motile-sessile switch. No difference in susceptibility was noted when *P. aeruginosa* PAO1 and mutant strains grown planktonically, were treated with the protein synthesis inhibitor tobramycin or the biocide hydrogen peroxide. Moreover, no difference in susceptibility was noted following treatment with norfloxacin (Fig. 14). Moreover, no difference in susceptibility was noted for *P. aeruginosa* wild type biofilms and *bfiS* mutant biofilms following treatment with tobramycin, norfloxacin, and hydrogen peroxide (Fig. 14). In contrast, *sagS* mutant biofilms were significantly more susceptible to hydrogen peroxide than wild type biofilms with hydrogen peroxide treatment resulting in a 3.5±0.2 log reduction in viability. Moreover, *ΔsagS* mutant biofilms were considerably more susceptible to tobramycin, resulting in a 3.5-log reduction and norfloxacin, resulting in a 2.5-log reduction. The same treatments only resulted in a ~0.2±0.1-log reduction in *P. aeruginosa* wild type biofilms and ~0.94±0.23 log reduction for *bfiS* biofilms, respectively. Overall, no difference in susceptibility was noted for a *sagS* mutant regardless of the mode of growth (planktonic or biofilm mode of growth).

The findings indicated that mutant biofilms arrested at or soon after the transition to the surface are more susceptible to antimicrobial agents, with *sagS* mutant biofilms being more susceptible than *bfiS* biofilms as compared to wild type biofilms. The findings furthermore suggests that SagS dephosphorylation is essential for biofilms to develop their resistance.

Since *plkR* biofilms were shown to have no biofilm defect (Fig. 10) and appeared to be comparable in susceptibility to wild type biofilms, we tested biofilms overexpressing *plkR* for differences in susceptibility to antimicrobial agents. Overexpression of plkR was shown to coincide with biofilm dispersion (Figs. 10-11). Inactivation or overexpression of plkR did not affectr susceptibility of cells grown planktonically (Fig. 15). However, biofilms overexpressing plkR were rendered more susceptible to tobramycin and hydrogen peroxide (Fig. 15). No difference in susceptibility to norfloxacin was observed (Fig. 15).

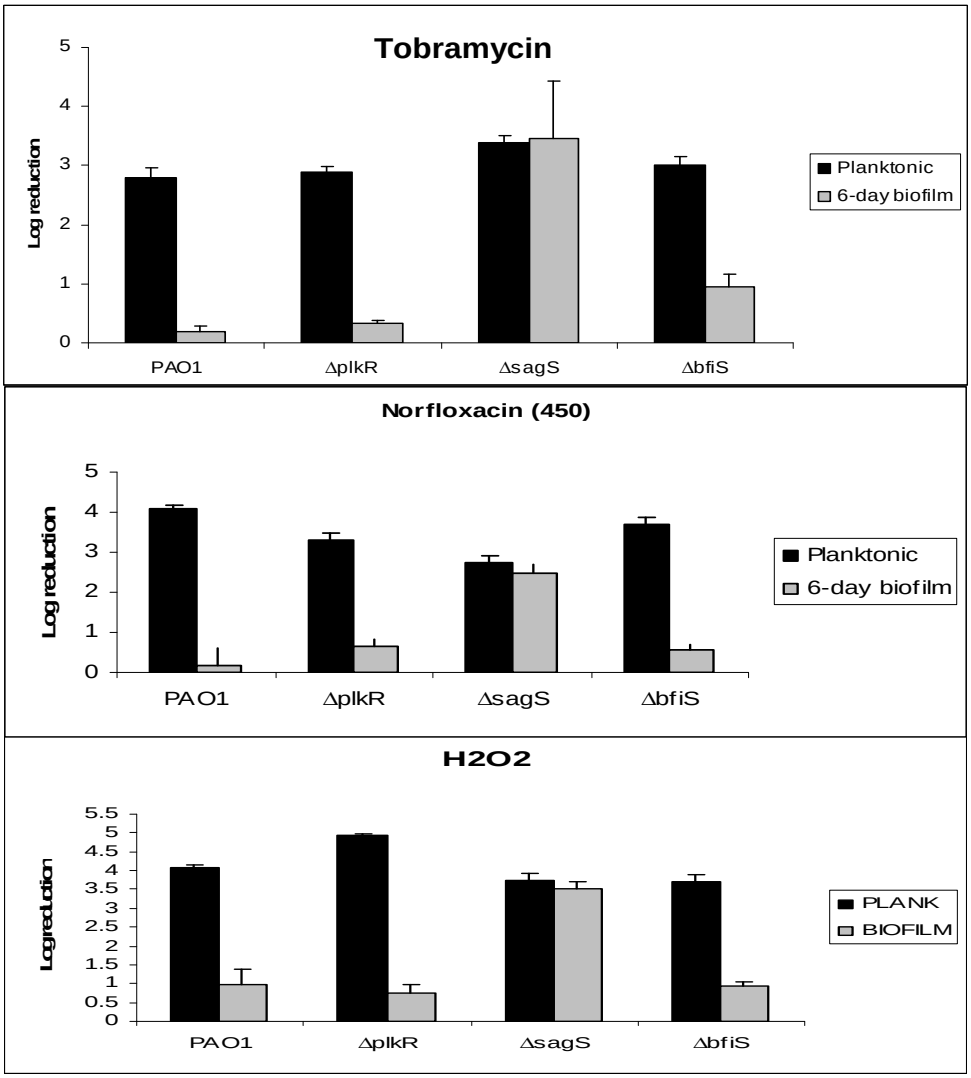

Fig. 14. Effect of inactivation of plkR, sagS and bfiS on susceptibility of planktonic and biofilm cells to tobramycin, norfloxacin and hydrogen peroxide.

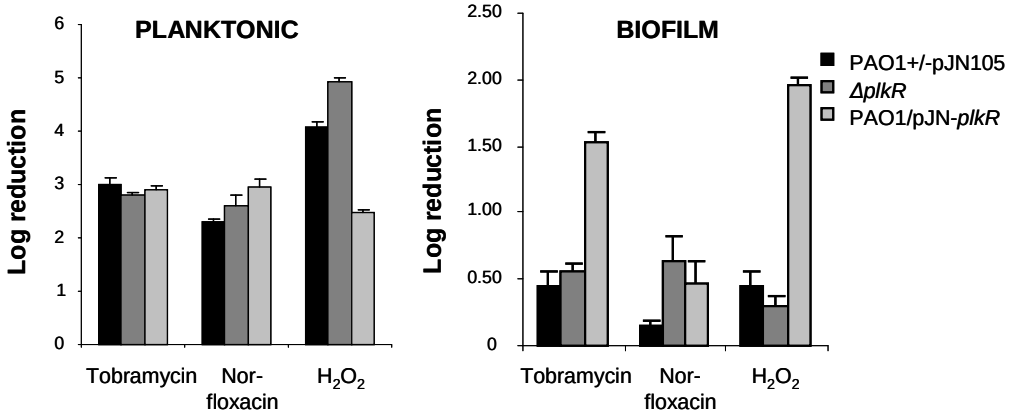

Fig. 15. Effect of inactivation and overexpression of *plkR* on susceptibility of planktonic and biofilm cells to hydrogen peroxide, norfloxacin and tobramycin.

Biofilms inactivated in *sagS*, *bfiS* or overexpressing *plkR* differentially express genes involved in conferring antimicrobial resistance.

Since many antibiotics are extruded from the *P. aeruginosa* cells via multidrug efflux pumps, transcript abundance of three efflux pumps represented by the genes *mexA*, *mexE* and *mexX* was analyzed by real-time qRT-PCR (Fig. 16). Moreover, expression of the Mercury resistance family regulator PA4878, the PhoPQ operon (see *phoP*), and PhoPQ- and PmrAB-regulated cationic antimicrobial peptide (CAP) resistance operon PA3552–PA3559 (*arnBCADTEF-ugd*, see PA3353) was analyzed (Fig. 16).

ΔsagS mutant biofilms expressed significantly less mexA, mexE, and mexX releative to *P. aeruginosa* wild type biofilms. Moreover, the mutant was found to also express less PA4878, phoP and PA3553 (Fig. 16), indicating that this mutant may also be more susceptible to cationic antimicrobial peptides. With the exception of *phoP* expression levels, similar results were obtained for the strain PAO1/pJN-plkR (Fig. 16). *ΔbfiS* mutant biofilms also expressed less *mexA*, PA3553, and PA4878, but in contrast to *ΔsagS* biofilms, overexpressed *mexE*. No difference in expression levels was noted for *phoP* and *mexX* (Fig. 16).

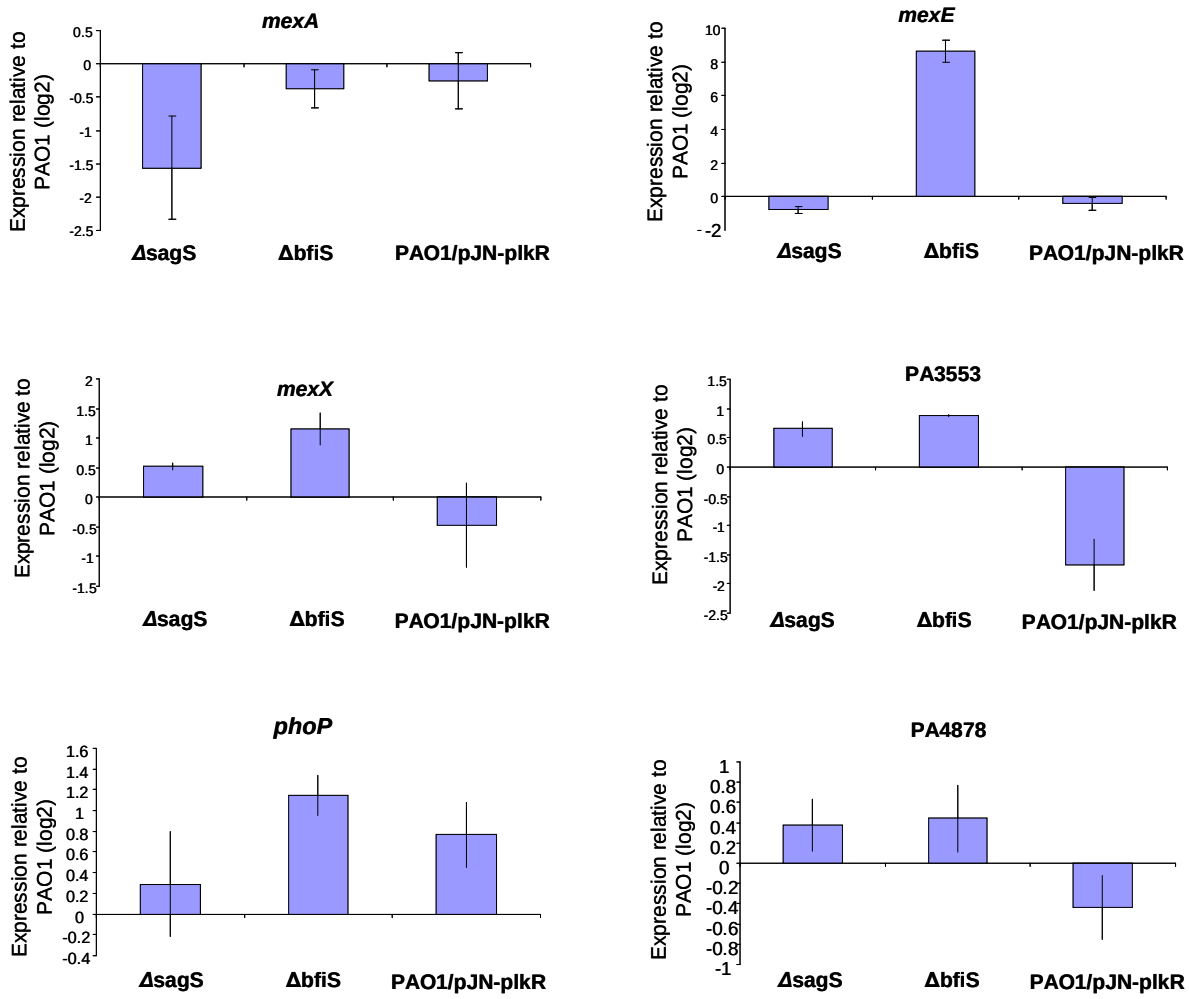

Fig. 16. Detection of differential expression of mexA, mexE, mexX, PA4878, phoP and the PhoPQ-regulated gene PA3553 in biofilms inactivated in *sagS*, *bfiS* or overexpressing *plkR*. Expression levels were determined relative to *P. aeruginosa* PAO1 biofilms by real-time qRT-PCR.

Virulence capabilities of mutant strains inactivated in *sagS, bfiS, and plkR.*

The above findings demonstrated an influence of regulators involved in the motile- e switch to play a role in the resistance phenotype of *P. aeruginosa* biofilms. This prompted us to test whether inactivation of regulatory proteins involved in biofilm development also play a role in virulence by using two distinct *in vivo* plant infection models. We chose this model of virulence as it as been previously shown that virulence outcomes obtained using this model can be correlated to outcomes obtained using the burned mouse pathogenicity model (Rahme *et al.*, 1995; Rahme *et al.*, 1997).

Using this model, *P. aeruginosa ΔbfiS* was avirulent as determined using the *Arabidopsis thaliana* infection model (plant death was used as indicator of virulence). While more than 44% of *Arabidopsis* plants were killed by *P. aeruginosa* PA14 (Fig. 17) within 8 days post infection, the isogenic *ΔbfiS* mutant was unable to establish an infection. The *ΔsagS* mutant strains was hypervirulent compared to the wild type causing 28% of plant death within 4 days (Fig. 17). In contrast, only 20% of all plants were killed by the wild type. *ΔplkR* displayed an intermediate phenotype indicated by the finding that *plkR* did not initially cause plant death (Fig. 17, day 4), but at later time points was comparable in virulence to the wild type (Fig. 17, day 8-10).

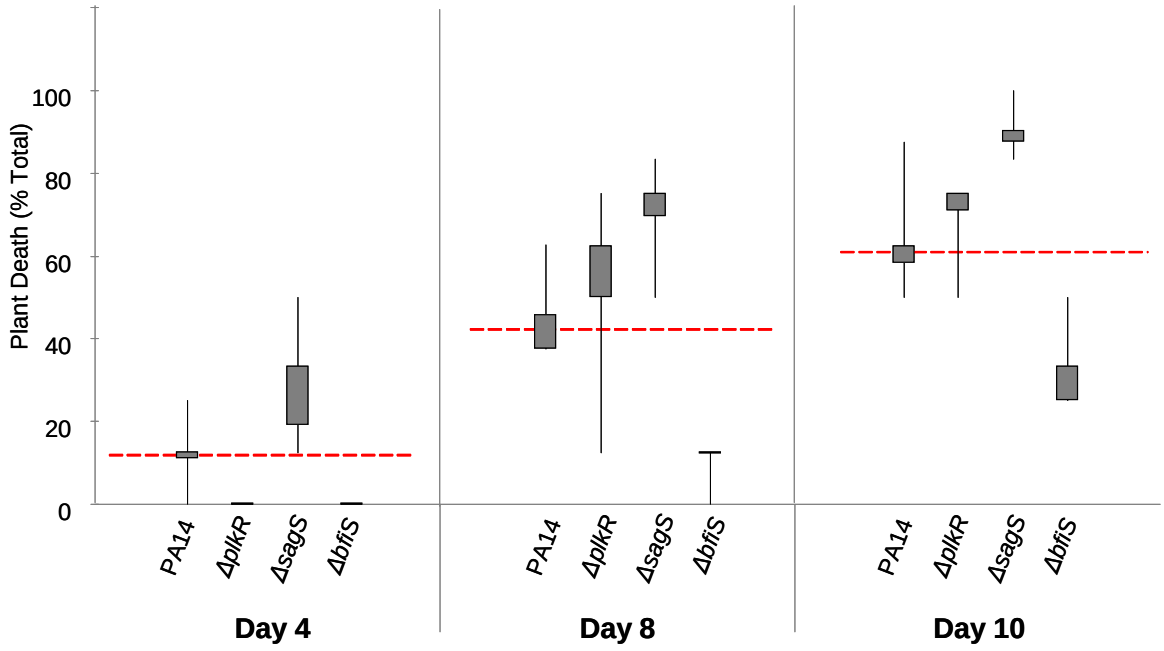

Fig. 17. Effect of *sagS, bfiS,* and *plkR* inactivation on *P. aeruginosa* virulence. *A. thaliana* plants, grown with the indicated PA14 strains, were monitored for signs of death manifesting as wilting and necrosis spread throughout the entire plant for 10 days. The graphs represent the averages of three experiments, with 8 plants used per replicate per strain. Lines indicate the difference between highest and lowest survival rates observed, while bars represent distribution between the mean and median of the replicates. Red dashed line indicates level of plant death by *P. aeruginosa* PA14 wild type.

CONCLUSION

The goal of the proposal was to identify biofilm-specific regulatory proteins involved in the regulation of initial biofilm formation that once inactivated, impair or arrest the biofilm formation process. Here, we have demonstrated that initiation of biofilm formation and the subsequent development of biofilms is controlled by at least two regulatory protein systems, the two-component regulatory system BfiSR and the two-component sensor hybrid SagS. The gene products of both regulatory systems are required for biofilm development and for maintenance of the biofilm architecture once biofilms have matured.

We have demonstrated that BfiSR and SagS form a signaling cascade required for biofilm development and that both two-component regulatory systems alter *rsmYZ* levels in a distinct and growth mode-dependent manner with modulation of *rsmY* being absolutely required for *P. aeruginosa* biofilm development. We furthermore demonstrated that PlkR is not required for biofilm formation but instead appears to play a role in the planktonic mode of growth. Based on our preliminary data, it appears that PlkR indirectly regulates the intracellular levels of the signaling molecule cyclic-di-GMP. Cyclic-di-GMP levels were found to be high in biofilms in the absence of *plkR* expression but were low in the presence of *plkR* expression.

We further demonstrated that inactivation does not only impair the progression of biofilm development but also result in biofilms that are more susceptible to antimicrobial agents than mature biofilms. In particular, inactivation of *sagS* rendered biofilms as susceptible to antimicrobial agents than the same cells grown planktonically. The finding indicates that SagS dephosphoryaltion plays an important role in the activation of genes that render biofilms resistance to antimicrobial agents. Here, we demonstrated that inactivation of *sagS* coincided with reduced expression levels of three efflux pumps, as well as PhoP and PhoPQ-regulated cationic antimicrobial peptide (CAP) resistance operon.

Taken together, our findings strongly suggest a signaling network composed of at least two regulatory protein systems, BfiSR and SagS that are not only essential for biofilm formation to occur in a developmental and stage-specific manner, but also for virulence and antimicrobial resistance. Both regulatory systems were also shown to be required for the maintenance of biofilms once they reached maturity. By interfering with the phosphorylation patterns of these regulatory systems, we could remove biofilms by causing established biofilms to collapse and thus, being more susceptible to antimicrobial treatment. Thus, interfering with phosphorylation patterns or the signaling cascade resulting in phopshorylation of BfiSR (or dephosphorylation of SagS), could lead to new ways of treating chronic infections. Taken together, our findings strongly suggest that the regulatory networks identified here (BfiSR, SagS, PlkR) have the potential to be used as target genes/proteins to arrest, control, and eradicate biofilms.

PUBLICATIONS resulting from this project

Published

*__Petrova, O.E., and K. Sauer.__ 2009. A Novel Signaling Network Essential for Regulating *Pseudomonas aeruginosa* Biofilm Development. PLoS Pathogens 5: e1000668.

** __Petrova, O.E., and K. Sauer.__ 2010. The novel two-component regulatory system BfiSR regulates biofilm development directly by controlling the small RNA *rsmZ* through CafA. J. Bacteriol. 192:5275-5288**.**

- * This publication received some media attention and excerpts were featured on the following websites:
  - http://www.sciencecodex.com/researchers_discover_new_ways_to_treat_chronic_infections
  - http://www.sciencedaily.com/releases/2009/12/091218151327.htm
  - http://www.healthjockey.com/2010/01/02/development-of-biofilms-may-lead-to-new-ways-for-treating-chronic-infections/
  - http://www.britannica.com/bps/additionalcontent/18/47256242/A-Novel-Signaling-Network-Essential-for-Regulating-Pseudomonas-aeruginosa-Biofilm-Development

** This publication was highlighted by a commentary in the *Journal of Bacteriology* (Goodman, 2010) as well by a journal highlight in the ASM journal *Microbe* (http://www.microbemagazine.org/index.php/10-2010-journal-highlights/2913-improved-understanding-of-regulation-of-biofilm-formation)

Manuscripts in preparation

Marques, C, O.E. Petrova, Y. Li, and K. Sauer. Antimicrobial resistance and virulence are developmental aspects of biofilm formation by the human pathogen *Pseudomonas aeruginosa.*

Petrova, O.E., and K. Sauer. SagS contributes to the sessile-motile switch by the human pathogen *Pseudomonas aeruginosa*

Petrova, O.E., and K. Sauer. Role of the transcriptional regulator PlkR in attachment and biofilm dispersion.

PRESENTATIONS resulting from this project

Oral presentations, invited

09/2010	Regulatory systems required for biofilm development and the maintenance of the planktonic mode of growth. Biofilms IV, 4th International SFAM meeting on Biofilms, Winchester, UK.
07/2010	Biofilm Development and Dispersion. SFAM Summer conference, Brighton, UK.
04/2010	Signaling networks essential for regulating *Pseudomonas aeruginosa* biofilm development, University of Oklahoma Health Sciences Center, Oklahoma City, OK.
04/2010	Signaling networks essential for regulating *Pseudomonas aeruginosa* biofilm development, Department of Microbiology, Montana State University, Bozeman, MT.

03/2010 A novel signaling network essential for regulating *Pseudomonas aeruginosa* biofilm development. Mathematical Biosciences Institute (MBI) workshop, Columbus, OH.

11/2009 A novel signaling network essential for regulating *Pseudomonas aeruginosa* biofilm development. 5th ASM (American Society for Microbiology) Biofilms conference, Cancun, Mexico

10/2009 A novel signaling network essential for regulating *Pseudomonas aeruginosa* biofilm development. Syracuse University, Syracuse, NY.

04/2009 Regulation of biofilm formation by *Pseudomonas aeruginosa.* Stevens Institute of Technology, Department of Engineering, Hoboken, NJ.

02/2009 Regulation of biofilm formation by *Pseudomonas aeruginosa.* University of Rochester, Department of Microbbiology and Immunology, Rochester, NY.

10/2008 Using tags and isotopes to track post-translational modifications during biofilm formation by *Pseudomonas aeruginosa.* Binghamton University, Department of Chemistry, Binghamton, NY.

Poster presentations at national and international conferences

11/2009 * Petrova, O., K. Sauer. Novel regulatory systems responsible for maintaining the planktonic lifestyle of the opportunistic human pathogen *Pseudomonas aeruginosa.* 5th ASM (American Society for Microbiology) Biofilms conference, Cancun, Mexico

09/2009 ** Petrova, O., K. Sauer. Coordinated signaling events regulate transition from initial attachment to maturation during *Pseudomonas aeruginosa* biofilm formation. Eurobiofilms 2009, Rome, Italy.

05/2009 Petrova, O., K. Sauer. *Pseudomonas aeruginosa* biofilm development is coordinated by a sequential series of signal transduction events. ASM conference, 109th General meeting, Philadelphia, PA.

07/2008 Petrova, O., G. Vazquez, and K. Sauer.*Pseudomonas aeruginosa* biofilm development is coordinated by multiple stage-specific transcriptional regulators. Corning Incorporated Science & Technology, Elmira, NY.

05/2008 Petrova, O., G. Vazquez, and K. Sauer. *Pseudomonas aeruginosa* biofilm development is coordinated by multiple stage-specific transcriptional regulators. ASM conference, 108th General meeting, Boston, MA.

*, poster presentation received ASM student travel award.

**, poster presentation received best poster award.

REFERENCES cited

Barraud, N., Schleheck, D., Klebensberger, J., Webb, J.S., Hassett, D.J., Rice, S.A., and Kjelleberg, S. (2009) Nitric Oxide Signaling in *Pseudomonas aeruginosa* Biofilms Mediates Phosphodiesterase Activity, Decreased Cyclic Di-GMP Levels, and Enhanced Dispersal. *J. Bacteriol.* **191**: 7333-7342.

Bordi, C., Lamy, M.-C., Ventre, I., Termine, E., Hachani, A., Fillet, S., Roche, B., Bleves, S., Méjean, V., Lazdunski, A., and Filloux, A. (2010) Regulatory RNAs and the HptB/RetS signalling pathways fine-tune *Pseudomonas aeruginosa* pathogenesis. *Molecular Microbiology* **76**: 1427-1443.

Brencic, A., and Lory, S. (2009) Determination of the regulon and identification of novel mRNA targets of *Pseudomonas aeruginosa* RsmA. *Molecular Microbiology* **72**: 612-632.

Brencic, A., McFarland, K.A., McManus, H., R., Castang, S., Mogno, I., Dove, S.L., and Lory, S. (2009) The GacS/GacA signal transduction system of *Pseudomonas aeruginosa* acts exclusively through its control over the transcription of the *RsmY* and *RsmZ* regulatory small RNAs. *Molecular Microbiology* **73**: 434-445.

Flemming, H.-C., Neu, T.R., and Wozniak, D.J. (2007) The EPS Matrix: The "House of Biofilm Cells". *J. Bacteriol.* **189**: 7945-7947.

Friedman, L., and Kolter, R. (2004) Genes involved in matrix formation in *Pseudomonas aeruginosa* PA14 biofilms. *Mol Microbiol* **51**: 675-690.

Goodman, A.L., Kulasekara, B., Rietsch, A., Boyd, D., Smith, R.S., and Lory, S. (2004) A Signaling Network Reciprocally Regulates Genes Associated with Acute Infection and Chronic Persistence in *Pseudomonas aeruginosa*. *Developmental Cell* **7**: 745-754.

Goodman, A.L. (2010) Sit and Stay a While: How BfiSR Controls Irreversible Attachment in *Pseudomonas aeruginosa* Biofilms. *J. Bacteriol.* **192**: 5273-5274.

Heurlier, K., Williams, F., Heeb, S., Dormond, C., Pessi, G., Singer, D., Camara, M., Williams, P., and Haas, D. (2004) Positive control of swarming, rhamnolipid synthesis, and lipase production by the posttranscriptional RsmA/RsmZ system in *Pseudomonas aeruginosa* PAO1. *J. Bacteriol.* **186**: 2936–2945.

Hsu, J.L., Chen, H.C., Peng, H.L., and Chang, H.Y. (2008) Characterization of the histidine-containing phosphotransfer protein B-mediated multistep phosphorelay system in Pseudomonas aeruginosa PAO1. *J Biol Chem* **283**: 9933-9944.

Jackson, K.D., Starkey, M., Kremer, S., Parsek, M.R., and Wozniak, D.J. (2004) Identification of psl, a Locus Encoding a Potential Exopolysaccharide That Is Essential for Pseudomonas aeruginosa PAO1 Biofilm Formation. *J. Bacteriol.* **186**: 4466-4475.

Kaga, N., Umitsuki, G., Nagai, K., and Wachi, M. (2002) RNase G-Dependent Degradation of the eno mRNA Encoding a Glycolysis Enzyme Enolase in *Escherichia coli Bioscience, Biotechnology, and Biochemistry* **66**: 2216-1110.

Kay, E., Humair, B., Denervaud, V., Riedel, K., Spahr, S., Eberl, L., Valverde, C., and Haas, D. (2006) Two GacA-dependent small RNAs modulate the quorum-sensing response in *Pseudomonas aeruginosa*. *J. Bacteriol.* **188**: 6026-6033.

Morgan, R., Kohn, S., Hwang, S.-H., Hassett, D.J., and Sauer, K. (2006) BdlA, a Chemotaxis Regulator Essential for Biofilm Dispersion in *Pseudomonas aeruginosa*. *J. Bacteriol.* **188**: 7335-7343.

Parkins, M.D., Ceri, H., and Storey, D.G. (2001) *Pseudomonas aeruginosa* GacA, a factor in multihost virulence, is also essential for biofilm formation. *Molecular Microbiology* **40**: 1215-1226.

Pessi, G., and Haas, D. (2001) Dual control of hydrogen cyanide biosynthesis by the global activator GacA in *Pseudomonas aeruginosa* PAO1. *FEMS Microbiol Lett* **200**: 73-78.

Petrova, O.E., and Sauer, K. (2009) A Novel Signaling Network Essential for Regulating *Pseudomonas aeruginosa* Biofilm Development. *PLoS Pathogens* **5**: e1000668.

Petrova, O.E., and Sauer, K. (2010) The novel two-component regulatory system BfiSR regulates biofilm development by controlling the small RNA *rsmZ* through CafA. *Journal of Bacteriology* **192**: 5275-5288

Rahme, L.G., Stevens, E.J., Wolfort, S.F., Shao, J., Tompkins, R.G., and Ausubel, F.M. (1995) Common virulence factors for bacterial pathogenicity in plants and animals. *Science* **268**: 1899-1902.

Rahme, L.G., Tan, M.-W., Le, L., Wong, S.M., Tompkins, R.G., Calderwood, S.B., and Ausubel, F.M. (1997) Use of model plant hosts to identify *Pseudomonas aeruginosa* virulence factors. *Proc Natl Acad Sci* **94**: 13245-13250.

Ryder, C., Byrd, M., and Wozniak, D.J. (2007) Role of polysaccharides in *Pseudomonas aeruginosa* biofilm development. *Current Opinion in Microbiology* **10**: 644-648.

Sauer, K., and Camper, A.K. (2001) Characterization of Phenotypic Changes in *Pseudomonas putida* in Response to Surface-Associated Growth. *J. Bacteriol.* **183**: 6579-6589.

Sauer, K., Camper, A.K., Ehrlich, G.D., Costerton, J.W., and Davies, D.G. (2002) *Pseudomonas aeruginosa* Displays Multiple Phenotypes during Development as a Biofilm. *J. Bacteriol.* **184**: 1140-1154.

Sauer, K., Cullen, M.C., Rickard, A.H., Zeef, L.A.H., Davies, D.G., and Gilbert, P. (2004) Characterization of Nutrient-Induced Dispersion in *Pseudomonas aeruginosa* PAO1 Biofilm. *J. Bacteriol.* **186**: 7312-7326.

Simm, R., Morr, M., Kader, A., Nimtz, M., and Romling, U. (2004) GGDEF and EAL domains inversely regulate cyclic di-GMP levels and transition from sessility to motility. *Molecular Microbiology* **53**: 1123-1134.

Southey-Pillig, C.J., Davies, D.G., and Sauer, K. (2005) Characterization of Temporal Protein Production in *Pseudomonas aeruginosa* Biofilms. *J. Bacteriol.* **187**: 8114-8126.

Tock, M.R., Walsh, A.P., Carroll, G., and McDowall, K.J. (2000) The CafA Protein Required for the 5'-Maturation of 16 S rRNA Is a 5'-End-dependent Ribonuclease That Has Context-dependent Broad Sequence Specificity. *J. Biol. Chem.* **275**: 8726-8732.

Umitsuki, G., Wachi, M., Takada, A., Hikichi, T., and Nagai, K. (2001) Involvement of RNase G in *in vivo* mRNA metabolism in *Escherichia coli*. *Genes to Cells* **6**: 403-410.

Williams, P., Winzer, K., Chan, W.C., and CÃ¡mara, M. (2007) Look who's talking: communication and quorum sensing in the bacterial world. *Philosophical Transactions of the Royal Society B: Biological Sciences* **362**: 1119-1134.

REPORT OF INVENTIONS AND SUBCONTRACTS

(Pursuant to "Patent Rights" Contract Clause) (See Instructions on back)

Form Approved
OMB No. 9000-0095
Expires Jan 31, 2008

PLEASE DO NOT RETURN YOUR COMPLETED FORM TO THE ABOVE ORGANIZATION. RETURN COMPLETED FORM TO THE CONTRACTING OFFICER.

1.a. NAME OF CONTRACTOR/SUBCONTRACTOR	c. CONTRACT NUMBER	2.a. NAME OF GOVERNMENT PRIME CONTRACTOR	c. CONTRACT NUMBER	3. TYPE OF REPORT *(X one)*
The Research Foundation of SUNY	W911NF-07-1-0604	US Army REDECOM ACQ CTR		a. INTERIM [ ] b. FINAL [X]

b. ADDRESS *(Include ZIP Code)*	d. AWARD DATE *(YYYYMMDD)*	b. ADDRESS *(Include ZIP Code)*	d. AWARD DATE *(YYYYMMDD)*	4. REPORTING PERIOD *(YYYYMMDD)*
P.O. Box 6000 Binghamton, NY 13902	20070917	4300 S. Miami Blvd Durham, NC 27703		a. FROM 20070917 b. TO 20100916

SECTION I - SUBJECT INVENTIONS

5. "SUBJECT INVENTIONS" REQUIRED TO BE REPORTED BY CONTRACTOR/SUBCONTRACTOR *(If "None," so state)*

NAME(S) OF INVENTOR(S) *(Last, First, Middle Initial)* a.	TITLE OF INVENTION(S) b.	DISCLOSURE NUMBER, PATENT APPLICATION SERIAL NUMBER OR PATENT NUMBER c.	ELECTION TO FILE PATENT APPLICATIONS *(X)* d.				CONFIRMATORY INSTRUMENT OR ASSIGNMENT FORWARDED TO CONTRACTING OFFICER *(X)* e.	
			(1) UNITED STATES		(2) FOREIGN			
			(a) YES	(b) NO	(a) YES	(b) NO	(a) YES	(b) NO
	(NONE)							

f. EMPLOYER OF INVENTOR(S) NOT EMPLOYED BY CONTRACTOR/SUBCONTRACTOR		g. ELECTED FOREIGN COUNTRIES IN WHICH A PATENT APPLICATION WILL BE FILED	
(1) (a) NAME OF INVENTOR *(Last, First, Middle Initial)*	(2) (a) NAME OF INVENTOR *(Last, First, Middle Initial)*	(1) TITLE OF INVENTION	(2) FOREIGN COUNTRIES OF PATENT APPLICATION
(b) NAME OF EMPLOYER	(b) NAME OF EMPLOYER		
(c) ADDRESS OF EMPLOYER *(Include ZIP Code)*	(c) ADDRESS OF EMPLOYER *(Include ZIP Code)*		

SECTION II - SUBCONTRACTS *(Containing a "Patent Rights" clause)*

6. SUBCONTRACTS AWARDED BY CONTRACTOR/SUBCONTRACTOR *(If "None," so state)*

NAME OF SUBCONTRACTOR(S) a.	ADDRESS *(Include ZIP Code)* b.	SUBCONTRACT NUMBER(S) c.	FAR "PATENT RIGHTS" d.		DESCRIPTION OF WORK TO BE PERFORMED UNDER SUBCONTRACT(S) e.	SUBCONTRACT DATES *(YYYYMMDD)* f.	
			(1) CLAUSE NUMBER	(2) DATE *(YYYYMM)*		(1) AWARD	(2) ESTIMATED COMPLETION
	(NONE)						

SECTION III - CERTIFICATION

7. CERTIFICATION OF REPORT BY CONTRACTOR/SUBCONTRACTOR *(Not required if: (X as appropriate))* [ ] **SMALL BUSINESS or** [ ] **NONPROFIT ORGANIZATION**

I certify that the reporting party has procedures for prompt identification and timely disclosure of "Subject Inventions," that such procedures have been followed and that all "Subject Inventions" have been reported.

a. NAME OF AUTHORIZED CONTRACTOR/SUBCONTRACTOR OFFICIAL *(Last, First, Middle Initial)*	b. TITLE	c. SIGNATURE	d. DATE SIGNED
Parker, Paul. C	Associate Vice President		20101203